

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



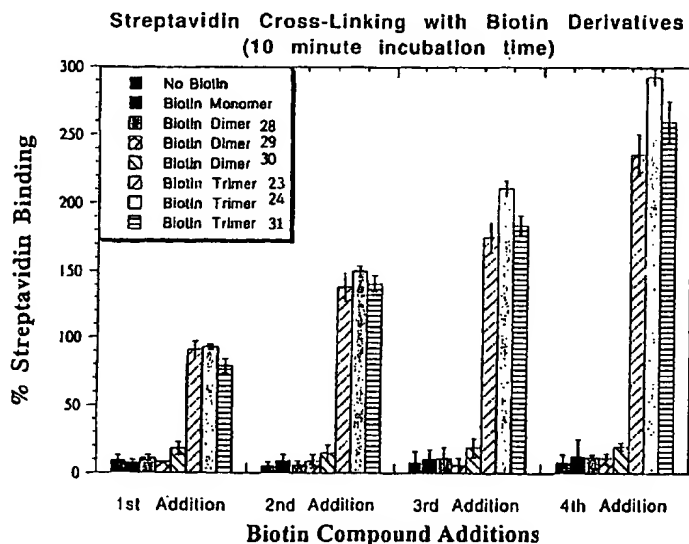
(43) International Publication Date
7 December 2000 (07.12.2000)

PCT

(10) International Publication Number
WO 00/72802 A2

- (51) International Patent Classification⁷: A61K (74) Agents: MUELLER, Jerry, K., Jr. et al.; Mueller and Smith, LPA, 7700 Rivers Edge Drive, Columbus, OH 43235 (US).
- (21) International Application Number: PCT/US00/15081
- (22) International Filing Date: 1 June 2000 (01.06.2000) (81) Designated States (*national*): AU, BR, CA, IL, JP, KR, MX, RU.
- (25) Filing Language: English (84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
- (26) Publication Language: English
- (30) Priority Data: 09/324,267 2 June 1999 (02.06.1999) US Published:
— Without international search report and to be republished upon receipt of that report.
- (71) Applicant: UNIVERSITY OF WASHINGTON For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
[US/US]; 1107 NE 45th Street, Seattle, WA 98105-4631 (US).

(54) Title: WATER SOLUBLE MULTI-BIOTIN-CONTAINING COMPOUNDS



(57) Abstract: Water soluble discrete multi-biotin-containing compounds with at least three (3) biotin moieties are disclosed. The water soluble biotin-containing compounds may additionally comprise one or more moieties that confer resistance to cleavage by biotinidase or that is cleavable *in vitro* or *in vivo*. The discrete multi-biotin-containing compounds may include a reactive moiety that provides a site for reaction with yet another moiety, such as a targeting, diagnostic or therapeutic functional moiety. Biotinylation reagents comprising water soluble linker moieties are also disclosed and may additionally comprise a biotinidase protective group. Methods for amplifying the number of sites for binding biotin-binding proteins at a selected target using multi-biotin compounds are also disclosed.

WO 00/72802 A2

WATER SOLUBLE MULTI-BIOTIN-CONTAINING COMPOUNDS

Reference to Priority Applications

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/798,413 filed February 7, 1997, entitled BIOTIN-CONTAINING COMPOUNDS, BIOTINYLATION REAGENTS AND METHODS, now abandoned, which claims
5 priority from U.S. Provisional Patent Application No. 60/011,321, filed February 8, 1996, entitled WATER SOLUBLE BIOTIN DERIVATIVES.

Field of the Invention

The present invention relates generally to discrete multi-biotin-containing
10 compounds, biotinylation reagents, and methods for synthesizing such compounds and reagents. The present invention relates, more specifically, to discrete biotin-containing compounds with at least three (3) biotin moieties, and biotinylation reagents suitable for *in vitro* and *in vivo* applications that are soluble in an aqueous media and preferably resistant to biotinidase. The biotin-containing
15 compounds may additionally comprise constituents that confer other functionalities, such as conjugation sites for diagnostic or therapeutic moieties. The compounds according to the invention may also comprise reporter moieties (e.g., fluorophores) that can be used to determine the number of multi-biotin species that have been attached to a functional moiety such as an antibody.

20

Background of the Invention

The very strong interaction of biotin with the proteins avidin and streptavidin renders biotin-containing compounds useful for numerous applications. For example, many diagnostic tests use biotinylated derivatives. The
25 widely used enzyme linked immunosorbent assay (ELISA), which was developed as an alternative to radioimmunoassays, employs biotinylated antibodies. Other biotinylated compounds have been used as probes and biotinylated nucleic acids have also been widely used. Purification techniques such as affinity chromatography frequently employ biotinylated materials.

30 More recently, biotin derivatives have been used in diagnosis and therapy of human disease. Notably, investigators have shown that use of a combination of monoclonal antibodies, streptavidin and/or avidin, and radiolabeled biotin

derivatives, improves the diagnostic and therapeutic characteristics of the radiolabeled monoclonal antibody tumor targeting system. An example would be the "pre-targeting" of monoclonal antibody conjugates using the biotin/(strept)avidin ligand/anti-ligand pair for imaging and therapy of cancer.

5 One disadvantage of using biotin derivatives for many of these applications is their generally low solubility in an aqueous media. In many examples, biotin derivatives and biotinylation reagents generally need to be solubilized in organic solvents or a medium comprising a substantial level of organic constituents to attain aqueous solubility. Insolubility of biotin derivatives
10 and biotinylation reagents in aqueous solutions is particularly problematic for *in vivo* applications where organic solvents cannot be used.

 It has been recognized that the availability of biotin derivatives for binding with avidin- and streptavidin-containing compounds may be greater when a spacer molecule is used between the biotin moiety and the other moieties to which
15 the biotin is attached. The spacer molecules previously used have generally possessed low solubility in aqueous medium. Such linkers reduce the aqueous solubility of the biotin-containing compound. In addition to causing solubility problems, the lipophilic nature of the biotin derivatives with linkers of low water solubility may cause them to associate with blood components, rendering their
20 biological half-life in *in vivo* applications longer than desired. Prior investigators have attached water solubilizing moieties to biotin and to compounds containing two biotin moieties, however, the prior art has failed to disclose or suggest biotin-containing compounds that comprise at least three (3) biotin moieties which are resistant to the enzyme biotinidase.

25

Description of the Background Art

 U.S. Patents 5,541,287 and 5,578,287 disclose compositions for use in pre-targeted delivery of diagnostic and therapeutic agents that employ biotin/avidin as the ligand/anti-ligand binding pair. These patents disclose that the 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetra acetic acid (DOTA)-biotin adducts have
30 desirable *in vivo* biodistribution and are cleared primarily by renal excretion. However, these patents only teach biotin-containing compounds of up to two (2) biotin moieties. These patents also report that the disclosed adducts are not stable in serum due to the presence of biotinidase. Poor *in vivo* stability therefore
35 limits the use of such conjugates in therapeutic applications.

 U.S. Patent No. 5,326,778 to Rosebrough discloses conjugates of biotin

and deferoxamine for radioimmunoimaging and radioimmunotherapy that are capable of binding metal ions to avidin or streptavidin. This reference fails to suggest a biotin-containing compound that contains at least three (3) biotin moieties and water-solubilizing moieties of 6 to 50 atoms in length.

5 U.S. Patent No. 5,482,698 to Griffiths discloses a method for the detection and therapy of lesions with biotin/avidin polymer conjugates. The preferred polymers of Griffiths are the starburst dendrimers or dextrans. This reference, however, does not suggest water soluble linker moieties of 6 to 50 atoms in length and biotinidase blocking groups in a discrete multi-biotin-containing compound.
10 This reference also fails to suggest how dendrimers and dextrans can be used to produce discrete, water soluble, multi-biotin-containing compounds.

U.S. Patent No. 5,521,319 to Huber et al. teaches that biotin compounds which comprise water soluble linkers can be conjugated to various moieties, however, there is no suggestion that three (3) or more biotin moieties be present
15 in the compound and that biotinidase blocking groups be incorporated to impart *in vivo* stability.

U.S. Patent No. 5,750,357 to Olstein et al. describes a detectable synthetic copolymer wherein one monomer is a binding agent for microorganisms and the second monomer is a detectable label or a binding site for a detectable label, such
20 as biotin. The polymerization of these two monomers results in a random copolymer that may contain three (3) or more biotin moieties. However, there is no suggestion or disclosure that each biotin moiety be linked to a water soluble moiety of 6 to 50 atoms in length, that is in turn linked to a cross linker compound that has at least tri-functionality such as benzene 1,3,5-tricarbonyl trichloride,
25 starburst dendrimers, cascade dendrimers, polylysine, polyglutamic and polyaspartic acids. The thermally initiated random polymerization disclosed in Olstein produces a reaction mixture that consists of numerous polymeric species with varying molecular weights and number of biotin moieties. Such random biotin-containing compounds have a very limited utility in diagnostic and
30 therapeutic applications. Further, Olstein does not address the need for a biotinidase blocking moiety.

Hnatowich et al., in "Investigations of Avidin and Biotin for Imaging Applications", *J. Nucl. Med.*, Vol. 28, pp. 1294-1302, No. 8 (1987) discloses a reaction product that comprised a biocytin-DTPA-biotin dimer. This reference
35 does not suggest a compound comprising at least three (3) biotin moieties, water soluble linker moieties of 6 to 50 atoms in length and biotinidase protective moieties.

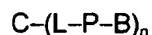
Lastly, biotinylated dextrans have been used as molecular probes for some time. See R.P. Haugland, "Handbook of Fluorescent Probes and Research Chemicals", Sixth Edition, Chapter 15.5, Molecular Probes, Inc., Eugene, Oregon. These probes are not discrete molecular entities and do not address the need for
5 resistance to biotinidase activity nor do they suggest the use of water soluble linker moieties of 6 to 50 atoms in length.

It is thus apparent that the prior art has paid little attention to the water solubility of biotin-containing compounds, or the synthesis of biotin reagents having three (3) or more biotin moieties and resistance to biotinidase. Because
10 biotin-containing compounds are of increasing interest for diagnostic and therapeutic applications, there is a need for enhanced biotin-containing compounds exhibiting excellent water solubility and improved resistance to cleavage by the serum enzyme biotinidase. The compounds according to the invention meet these needs, in part, through the selection of water soluble linkers
15 that allow for the optimum binding of the biotin moiety to avidin/streptavidin.

Summary of Invention

One aspect of the present invention is directed to discrete multi-biotin-containing compounds that are useful *in vivo* to amplify a signal or increase the
20 amount of material bound to a site. As used herein, the term "discrete" means a singular molecular entity and not a mixture of molecular entities (such as those resulting from a random polymerization reaction or a random labeling reaction). For example, the amount of radioactivity presented to a cancer cell that has been labeled with an antibody can be greatly increased through the use of multi-biotin-
25 containing compounds according to the invention.

Thus, there is disclosed a biotin-containing compound of the structural formula:

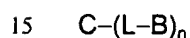


wherein C is selected from cross-linkers with at least tri-functionality; L is
30 selected from water soluble linker moieties of 6 to 50 atoms in length; P is a biotinidase protective group selected from α -amino acids, N-methyl and α -methyl moieties that block biotinidase activity; B is selected from biotin moieties; and n ranges from 3 to 64 and more preferably ranges from 3 to 32.

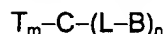
As used herein and in the claims, the term "cross-linkers" (C) means a
35 moiety selected from the group consisting of at least tri-functional aromatic compounds (e.g., benzenes), starburst dendrimers and cascade dendrimers.

Representative cross-linkers of at least tri-functionality useful in the present invention include polycarboxyl and polyamine compounds with 3 to 64 functional groups, discrete polylysine and polyglutamic and polyaspartic acids. As used herein and in the claims, the term "water soluble linker" (L) means a moiety of 6 to 50 atoms in length comprising one or more moieties selected from the group consisting of ethers, hydroxyls, amines, thioethers and thiols. As used herein and in the claims, the term "biotinidase protective group" (P) means an α -amino acid, N-methyl or α -methyl containing moiety that reduces the effectiveness of biotinidase to cleave the biotin moiety from the molecule of the invention. As used herein and in the claims, the term "biotin moiety" (B) means biotin and modified biotins such as desthiobiotin, biotin sulfone, iminobiotin, alkylated biotins, acetylated biotins and the like.

There is also disclosed a biotin-containing compound of the structural formula:

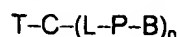


wherein C, L, B and n are defined as above. There is further disclosed a biotin-containing compound of the structural formula:



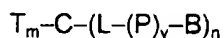
wherein C, L, B and n are defined as above, m is 1 to 5; and T is selected from a therapeutic, diagnostic or other active moieties, such as targeting molecules. Representative "T" moieties include those chemical entities that: 1) carry diagnostic and/or therapeutic radionuclides; 2) are diagnostic and/or therapeutic photoactive molecules; 3) are chemotherapeutic agents, or precursors or prodrugs thereof; 4) are protein toxins or derivatives thereof; 5) target tumors through a variety of receptor modalities; 6) bind with biologically active proteins; 7) cause the molecule to be excluded from cells; 8) allow the molecule to enter cells; and 9) cause it to target infections. More specifically, the "T" moiety may be, for example, a molecule chelated to or bound with a radionuclide (e.g. I-123, I-125, I-131, In-111, Y-90, At-211, Bi-213, etc), a photoactive group (e.g., dansyl, fluorescein, cyanocobalamin, cyanine dye, porphyrin, etc.), a drug (e.g., dehydrotestosterone, adriamycin, vincristine, 5-fluorouracil, etc.), a cancer targeting agent (e.g. monoclonal antibody or fragment, growth factor, signaling peptide, etc.), a MRI active agent (e.g., chelated gadolinium (Gd), fluorinated compounds, boron-11 compounds, etc), a neutron activated molecule (e.g., boron-10 containing molecules, Gd containing molecules, etc.), or a membrane crossing agent (e.g., transferrin, folate, cyanocobalamin, etc.).

There is also disclosed a functionalized biotin-containing compound of the structural formula:



wherein T, C, L, P, B and n are defined as above.

5 There is further disclosed a multi-biotin-containing compound of the structural formula:



wherein C is a cross-linker of at least tri-functionality selected from the group consisting of discrete tri-functionalized aromatic compounds, polycarboxylic
10 compounds, polyamino compounds, starburst dendrimers and cascade dendrimers; L is a water soluble moiety of 6 to 50 atoms in length that comprises one or more moieties selected from the group consisting of ethers, hydroxyls, amines, thioethers and thiols; P is a biotinidase protective group selected from α -amino acids, N-methyl and α -methyl moieties; y is 0 or 1; B is selected from biotin
15 moieties; n can range from 3 to 64, preferably 3 to 32; T is a therapeutic or diagnostic moiety; and m can range from 1 to 5.

In preferred embodiments of the invention, the water soluble linker moieties comprise 4,7,10-trioxa-1,13-tridecanediamine and 12-N-methylamino-4,7,10-trioxadodecanoic acid. As disclosed above, the biotin-containing compound may
20 also comprise diagnostic and therapeutic moieties joined to the compound.

Also disclosed is a non-polymerized biotin-containing compound comprising at least three (3) biotin moieties joined by water soluble linker moieties to a dendrimer, wherein said linker moieties are 8 to 20 atoms in length.

In yet another embodiment, the present invention discloses a novel
25 biotinylation reagent that may be used to prepare the multi-biotin-containing compounds of the invention. The biotinylation reagent has the structural formula:



wherein B, P and L are defined as above and X is a reactive functionality or a precursor thereof. Representative reactive functionalities include, but are not
30 limited to, nucleophilic groups such as amines, sulfhydryl groups or alkoxides and electrophilic groups such as activated carboxylates, mixed anhydrides, isothiocyanates, isocyanates, ketones, aldehydes, α,β -unsaturated ene-one moieties, alkyl halides, benzyl halides and similar functional groups with or without protecting moieties on them, these chemical protecting moieties are not the
35 enzymatic protective groups "P" as defined herein. Activated carboxylates include N-succinimidyl esters, tetrafluorophenyl esters and similar moieties as

described in the literature. The chemical protecting groups include t-butyloxycarbonyl (t-Boc), t-Bu esters, benzyloxycarbonyl (Z), phthalimidio, fluorenylmethyloxycarbonyl (Fmoc) and similar moieties described in the literature.

The present invention also relates to a biotin-containing compound
5 comprising at least three (3) biotin moieties joined to at least three water soluble linker moieties of 8 to 20 atoms and a cross-linking agent of at least tri-functionality. In a preferred embodiment, the water soluble linker moiety comprises one or more moieties selected from the group consisting of polyether, polyhydroxyl and polyamino moieties.

10 In a preferred embodiment, the multi-biotin-containing compound; has a water solubility greater than 0.2 mg/mL at neutral pH and ambient temperatures. Still more preferred, the multi-biotin-containing compound has a water solubility greater than 1 mg/mL at neutral pH and ambient temperature and most preferably a water solubility greater than 5 mg/mL.

15 As thus disclosed, the multi-biotin-containing compounds of the present invention are prepared by reacting a biotin molecule with water soluble linker moieties to form biotin/water soluble linker adducts. The biotin/water soluble linker adduct preferably also comprises a biotinidase protective group between the biotin moiety and the water soluble linker. This molecule is then bonded to a
20 cross-linker that possesses at least tri-functionality to result in a discrete molecular entity that comprises three (3) or more biotin moieties. Such biotin-containing compounds preferably comprise one or more protective groups that confer resistance to cleavage by biotinidase and a reactive or functional moiety that provides a site for reaction with yet another moiety, such as a targeting,
25 diagnostic or therapeutic moiety.

Brief Description of the Drawings

The invention will be described below in with reference to the following detailed description and Examples, and the figures in which:

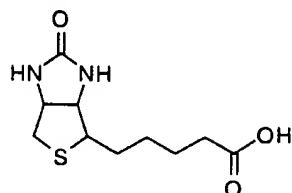
30 Fig. 1 illustrates the results of the experiment described in Example 7 demonstrating the successful cross-linking of streptavidin with biotin trimers; and

Fig. 2 illustrates the results of the experiments described in Example 12, demonstrating the successful cross-linking of streptavidin with biotin dendrimers.

Detailed Description of the Invention

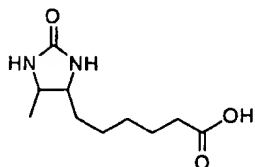
35 Naturally-occurring biotin, illustrated below as structure 1, in conjugated

form is useful for many different applications as a result of its strong binding affinity with the proteins avidin and streptavidin.

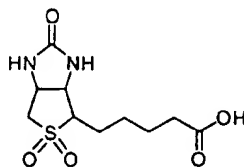


1

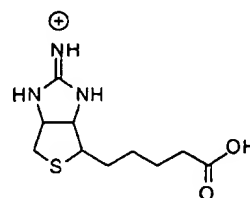
Many modified forms of biotin have been synthesized for various applications and are useful in the present invention as the biotin moiety (B) in addition to natural biotin. Modifications of biotin at positions other than the carboxylate group, for example, provide molecules that have weaker interactions with avidin or streptavidin. Such modified biotin molecules, such as desthiobiotin, shown below as structure 2, and biotin sulfone shown below as structure 3, are useful for some applications because they bind tightly enough to provide a strong association, yet they bind in a reversible fashion such that they can be displaced by tighter binding biotin derivatives (e.g., structure 1). Other modifications of biotin, such as conversion of the ureido functionality to a guanidinium functionality (e.g. iminobiotin, shown below (structure 4) or conversion of the amide-NH by alkylation (e.g. methylation) or by acylation (e.g., acetyl) produce biotins of varying binding strengths and can be the biotin moiety (B) of the compounds of the present invention.



2



3



4

Modifications of biotin or biotin derivatives by reaction with amines of sterically bulky groups such as proteins (e.g. insulin) or sterically small molecules such as branched chain moieties including 2-dimethyl-amino compounds or amino-aryl moieties are also advantageous for some applications. Preferred steric moieties that can be conjugated with the carboxylate of biotin, or a modified biotin, include substituted and unsubstituted aminobenzoic acids, or aminomethylbenzoic acids. As stated above, the "biotin moiety (B)" includes naturally occurring biotin and the modifications of naturally occurring biotin that bind to avidin, streptavidin, modifications and constituents thereof.

The solubility of naturally-occurring biotin in water is approximately 0.2 mg/mL at neutral pH and ambient temperature. Prior art modified biotins generally have a lower water solubility than natural biotin, and the prior art biotin compounds having more than one modified biotin moiety per molecule are generally substantially insoluble in aqueous media. For example, a prior art biotin compound comprising two biotin moieties joined by an aliphatic 1,12-diaminododecane linker moiety has such low water solubility that routine HPLC analysis does not detect any dimers in aqueous solution.

The solubility of biotin moieties in aqueous media is enhanced according to the present invention by attaching a water soluble linker moiety, typically through the carboxylate group of the biotin. Water soluble linker moieties (L), according to the present invention, comprise any linker moiety that, when coupled to a biotin moiety and the cross linker (C), increases the water solubility of the biotin-containing compound. The biotin-containing compounds of the present invention typically have aqueous solubility of greater than 0.2 mg/mL at neutral pH and ambient temperatures. Biotin-containing compounds and biotinylation reagents of the present invention preferably exhibit a water solubility of at least about 1 mg/mL at neutral pH and ambient temperature, and most preferably exhibit a water solubility of at least about 5 mg/mL at neutral pH and ambient temperature. Solubility may be ascertained by dissolving the compound in water, stirring the solution, and allowing the solution to stand at room temperature for about 24 hours. The solution is then centrifuged and the resultant aqueous layer analyzed using high performance liquid chromatography ("HPLC"). The HPLC analysis is conducted with a gradient using acetonitrile or methanol and water as the solvent mixture on a reversed-phase column.

Water soluble linker moieties (L) preferably comprise hydrophilic moieties (e.g., polar functional groups) including electronically neutral and charged (e.g., ionic) moieties. Suitable hydrophilic moieties include electronically neutral moieties containing polar functional groups (i.e., groups that contain atoms of differing electronegativity such as organic compounds containing nitrogen, oxygen, and sulfur) that increase their hydrophilicity. Typically, these neutral hydrophilic moieties contain functional groups that hydrogen bond with water. Such hydrogen bonding groups include ether (-O-), hydroxy (-OH), amino (-NR₂, -NHR, -NH₂), and to a lesser extent thioether (-S-), and thiol (-SH) groups.

Other polar functional groups that may serve as hydrophilic moieties include ethers and carbonyl-containing groups such as acids, esters, amides, ketones, and aldehydes. Moieties that comprise multiple polar functional groups

are more hydrophilic than those moieties that comprise a single polar functional group. Suitable moieties comprising multiple polar groups include, for example, polyhydroxyl, polyamido, polyether, polyphosphoric acid, polyalcohol and polyamine moieties. The polyhydroxyl moieties include, for example, glycols, glycerols, and polysaccharides including glucose, fructose, galactose, idose, inositol, mannose, tagatose, and N-methylglucamine. Polyether moieties include, for example, polyethylene glycol, penta(ethylene glycol), tetra(ethylene glycol), and tri(ethylene glycol). Polyamine moieties include, for example, polylysine, spermine or spermidine. Representative polycarboxylates include polyglutamic acid and polyaspartic acid.

Suitable charged hydrophilic moieties become either formally negatively or positively charged in water. Suitable negatively charged moieties include acid anions resulting from the dissociation of acids in water. For example, carboxylic acids (CO_2H) dissociate to form negatively charged carboxylate ions (CO_2^-) at pH greater than about 5. Other stronger acids such as phosphoric (H_3PO_4) and sulfonic (H_2SO_3) acids ionize to form phosphate (PO_4^{3-}) and sulfonate (SO_3^{2-}) anions, respectively, at pH greater than about 2. Other more weakly acidic moieties, such as phenols and thiols, may also dissociate to form their corresponding anionic derivatives that are also water solubilizing. Other suitable negatively charged moieties include monocarbon carboranes, *nido*-carboranes, decaboranes, and dodecaboranes.

Depending upon the pH of the aqueous solution, abasic moieties may become formally positively charged moieties in water. These moieties become highly water soluble through protonation in aqueous solution. For example, at a pH lower than about 8, amines (NR_2 , NHR_2 , NH_2) become ammonium ions (NHR_2^+ , NH_2R^+ , NH_3^+), which are water soluble moieties. Quaternary ammonium moieties ($-\text{NR}_3^+$) are water soluble at all pHs. Suitable charged solubilizing moieties also include polylysine groups.

Water soluble linkers are preferably relatively linear molecules greater than 4 atoms in length, preferably between 6 and 50 atoms in length, and most preferably about 8 to 20 atoms in length. In one preferred embodiment, the linker is a linear molecule of 12 to 15 atoms in length. In the context of the present invention, the term "atom" refers to a chemical element such as C, N, O, S, or the like. The number of atom ranges provided herein are based on the relatively linear accounting of the water soluble linker. One of ordinary skill in the art will appreciate that a linker may be linear, branched or ring structures, and may include combinations of these features.

Suitable water soluble linkers comprise at least two coupling or reactive groups allowing the linker to bind to both a biotin moiety (with or without the protective group P) and at least the tri-functional cross-linker (C). Empirical factors such as the size (e.g., molecular weight and molecular conformation) and
5 the nature (e.g., charge and constituents) of the water soluble linker moiety will be dictated by the specific application of the biotin-containing compound, e.g., therapeutic or diagnostic.

The cross-linker (C) may provide, in addition to the binding sites for the water soluble linkers, one or more binding sites for one or more functional or
10 target moieties. One of ordinary skill in the art will appreciate that this allows for any number of different molecules to couple with the biotin-containing compound, including, for example, markers, such as radiolabeled and fluorescent molecules; proteins and peptides, such as antibodies; and conjugating molecules. Suitable, at least tri-functional linkers include, but are not limited to benzene 1,3,5-tricarbonyl
15 trichloride, starburst dendrimers, cascade dendrimers, polyamine compounds and polycarboxyl compounds.

Two principal types of water soluble linker moieties (L) are preferred for use in the biotin-containing compounds and biotinylation reagents of the present invention. One type is a non-ionized water soluble linker, which is made more
20 soluble by functional groups such as ethers or hydroxyl groups. Non-ionized linker moieties render the biotin-containing compound more water soluble, while retaining the neutral character of the biotin moiety. Particularly advantageous non-ionized linker moieties comprise chains with at least one ether moiety in them, and which are terminated with amines, carboxylic acids, thiols, hydroxyl groups or
25 combinations of those functionalities. Examples of non-ionized soluble linker moieties are the commercially available molecules 4,7,10-trioxa-1,13-tridecanediamine; or 2,2'-(ethylenedioxy)diethylamine, and tetraethylene glycol. Thioether containing linker moieties are also advantageous in this application. It should be recognized that after conversion to the water soluble linker (L), there
30 will be no ionizable feature in the molecule.

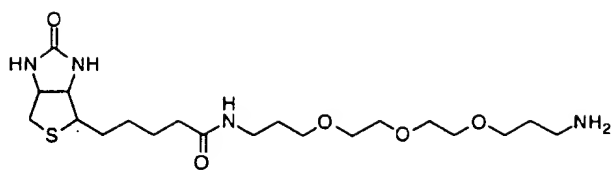
In the preparation of the biotin-containing compounds according to the invention, the biotin moiety (B) is typically activated at a selected coupling site (e.g., carboxylic group) and the water soluble linker (L) is then coupled to the biotin moiety to form a biotin moiety/water soluble linker adduct. This adduct can
35 be prepared using any one of several means, including, for example, an amide forming reaction, employing an amine group on the linker and the carboxylate coupling site on the biotin moiety. Alternatively, a water soluble linker may be

coupled to a biotin moiety through an amide forming reaction employing a carboxylate group on the water soluble linker and an amino group on the biotin moiety. It should be noted that after forming the biotin/water soluble linker adduct (B-L), that this reaction product is then reacted with the cross linker (C). As
5 discussed below, it is sometimes advantageous to place a biotinidase blocking moiety (P) between B and L, (thus, B-P-L).

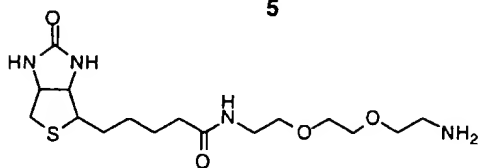
The amide reaction to form the biotin/water soluble linker adduct may include the use of coupling agents. Suitable coupling agents include carbodiimide coupling agents, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
10 hydrochloride (EDC), 1-benzyl-3-(3-dimethylaminopropyl)carbodiimide (BDC), 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide (CMC), and 1,3-dicyclohexylcarbodiimide (DCC). Other suitable techniques are described in detail in Bodanszky, "Principles of Peptide Synthesis", *Springer Verlag*, Berlin, 1984.

Generally, non-ionized water soluble ether linking moieties can be bonded
15 to biotin through the carboxylate (or activated carboxylate) of a biotin moiety or through conjugation wherein the biotin carboxylate functionality has been reduced and activated towards reactions with nucleophiles. Detailed syntheses of representative biotin-containing compounds are set forth in the Examples below.

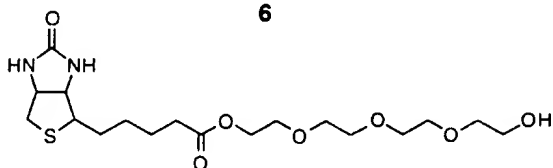
Some examples of useful biotin/moiety water soluble linker adducts are
20 shown below as structures 5-9.



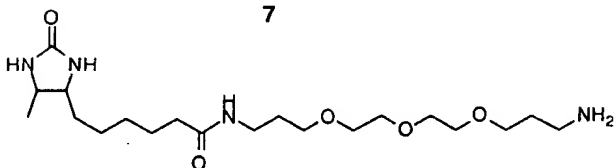
5



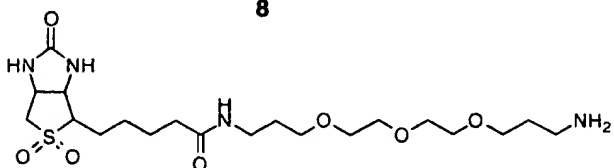
6



7

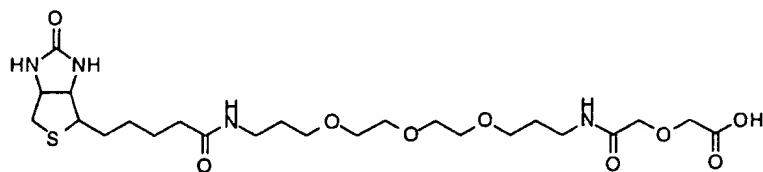


8

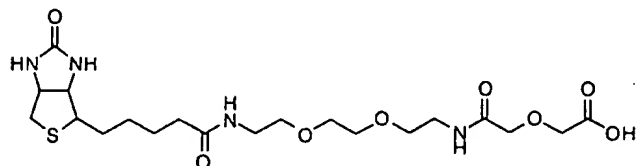


9

The water soluble linker moieties may also contain other functional groups attached to or within the chain (e.g., amides) as illustrated in structures **10** and **11** and may be synthesized in a sequential step of reactions.



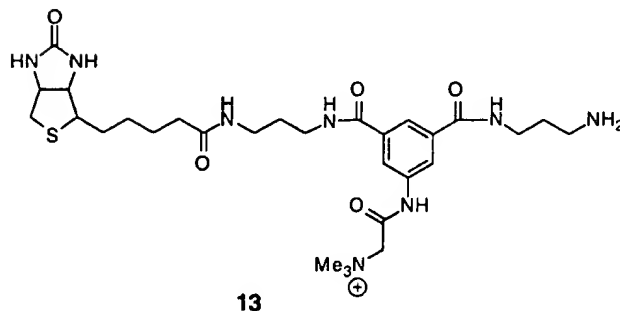
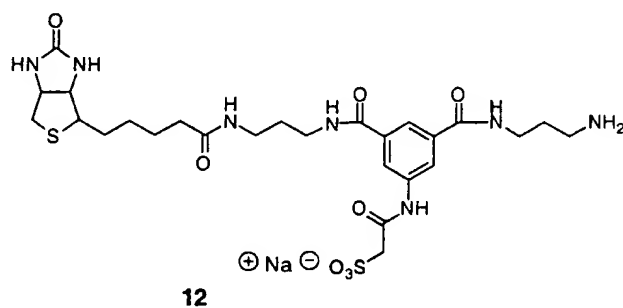
10



11

A second type of water soluble linker moiety comprises an ionized or ionizable moiety. The ionized functionality is preferably at least three (3) atoms away from the point of conjugation with the biotin moiety. Functional groups containing a sulfonate or an ammonium ion are advantageous. Linker moieties comprising anionic borane and carborane cage molecules are especially preferred, since these moieties provide biotin-containing compounds having enhanced water solubility and a site for radiolabeling.

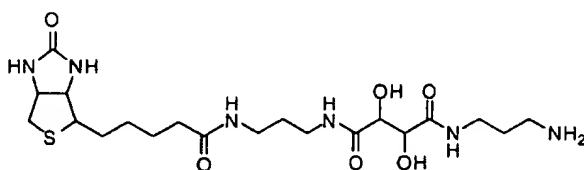
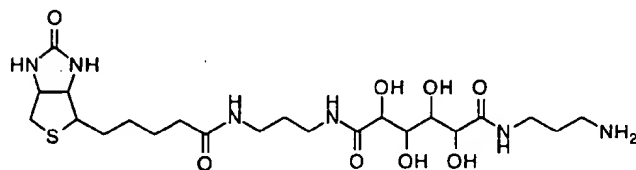
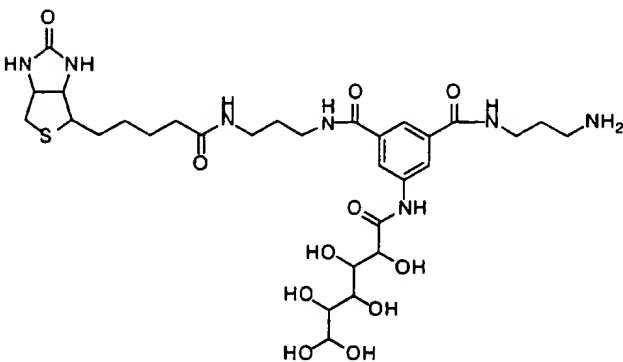
Representative biotin/water soluble linker adducts comprising ionic or ionizable functionalities are illustrated in structures 12 and 13 below:



Other combinations of ionic or ionizable functionalities and water soluble linker moieties may be used in combination with differing lengths of chains containing the ionic moiety branched from the water soluble linker moiety.

Useful ionic water soluble linker moieties include those that contain anionic boron cage moieties, such as a dodecaborane (icosahedral) cage moiety, which has a minus 2 (-2) charge and a *nido*-dicarbon carborane cage moiety, which has a minus 1 (-1) charge. Other anionic boranes or carboranes (*closo* or *nido*) may be used as water soluble linkers as well. The borane cage water solubilized linkers are useful when it is desirable to radiohalogenate the biotin-containing compound.

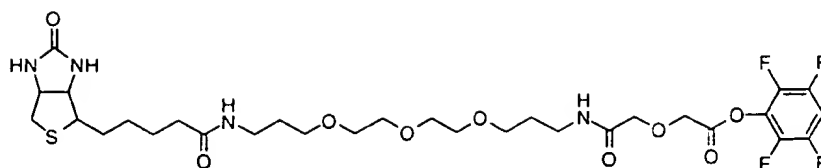
The compounds illustrated in structures **14-16** are exemplary of biotin/water soluble linker adducts that comprise polyhydroxyl moieties. These adducts may comprise from about 2 to 20 hydroxyl moieties. The hydroxyl groups may be bonded to the linking chain itself (as shown below in structures **14** and **15**) or are bonded to a branch point of the linker (as exemplified in structure **16**).

**14****15****16**

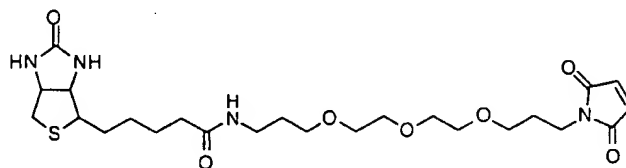
Modification of the biotin/water soluble linker adduct is sometimes desirable to prevent the serum enzyme biotinidase from cleaving the water soluble linker

from the biotin. This is one preferred embodiment of the invention. Introduction of a steric group alpha to the amine (or another functionality) of the water soluble linker which is attached to the biotin carboxylate group provides resistance to cleavage by biotinidase. Suitable steric moieties include carboxylates, larger alkyl groups, aryl groups, heteroaryl groups and other groups in the same manner. Depending upon the steric bulk of the branching group alpha to the amine (or other) functionality, attached to the carboxylate, some reduction in binding affinity for biotin-binding proteins may result. The particular application of the biotin-containing compound determines how much steric bulk is desired or can be tolerated in the branched group. Water soluble linkers possessing a branched chain alpha methyl (or other steric) group to the biotin moiety have been found useful in reducing *in vivo* degradation by biotinidase. Preferred α -methyl group containing linkers include 3-aminobutyric acid, 1,2-diaminopropane, and 1,4-diaminohexane (Dytek A). As will be described below, amino acids and N-methyl groups have been found useful in retarding biotinidase activity against the compounds of the invention.

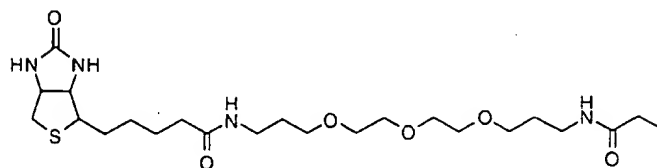
After preparation of the biotin/water soluble linking adduct, it is activated to form the biotinylation reagent according to the invention of structural formula B-P-L-X. B-P-L-X is then reacted with a cross-linker (C) that possesses at least tri-functionality. Exemplary activated biotin/water soluble linker adducts that can be reacted with a cross linker (C) include an activated ester (e.g., tetrafluorophenyl), **17**; maleimide, **18**; iodoacetamide, **19**; hydroxyl amine, **20**; acyl hydrazine, **21**; and nitrophenylazide, **22**. Additional reactive functionalities are described by Wilbur in *Bioconjugate Chem.*, 3, 433-470 (1992) and Bodanszky, "Principles of Peptide Synthesis", Chapter II, pp. 28-35, Springer-Verlag, New York, 1984.



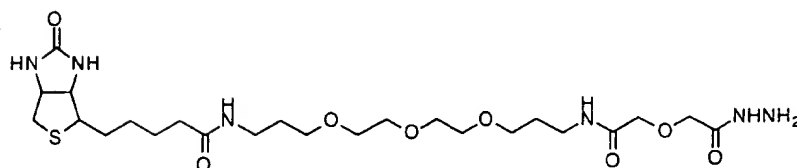
17



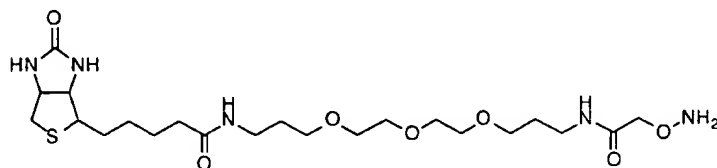
18



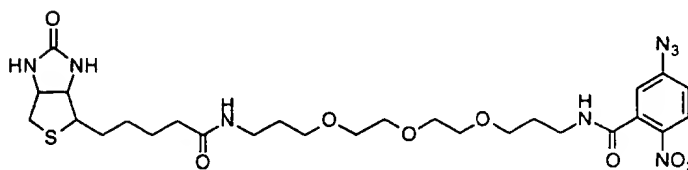
19



20



21



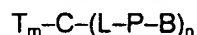
22

As disclosed above, the biotin-containing compounds of the present
5 invention may be linked to targeting moieties such as monoclonal antibodies, or
fragments or constituents thereof. For example, a combination of an intact
monoclonal antibody or a fragment (e.g., F(ab')₂, Fab', Fab, scFv, scFv₂) with

biologically produced, genetically engineered and/or chemically modified avidin, deglycosylated avidin, or streptavidin, can form a target for the biotin-containing compounds of the invention and used in *in vitro* assays, or for diagnostic and therapeutic *in vivo* applications. An important factor in these applications is that
5 the biotin-containing compounds of the present invention are highly water soluble, and this is important since binding proteins are most stable in aqueous media. More specifically, the multi-water soluble biotin-containing compounds according to the present invention can be linked to diagnostic or therapeutic functional moieties and used in *in vitro* assays or for *in vivo* applications for imaging and/or
10 therapy of human disorders (e.g., cancer, blood clotting, myocardial infarcts and the like).

Another application for the water soluble biotin-containing compounds according to the invention is to provide a targeting system that can be used with therapeutic drugs. Targeting of any number of therapeutic drugs to sites such as
15 tumors, can be accomplished with this system and biotinylated therapeutic drugs can be released at a selected site. It is important, in many instances, to release the drug from the biotin-containing compound as its most active form in the cell. This may be accomplished by introducing one or more cleavable functional groups at the point of attachment of the drug to the biotin-containing compound.

20 When containing a drug or other active moiety, the biotin-containing compound of the present invention has the structural formula:



wherein C, L, P, B and n are defined as above and T is the drug or other active moiety and m is 1 through 5. The drug may be linked to the cross linker (C)
25 through a bond that is cleaved at a pre-selected site, such as a tumor.

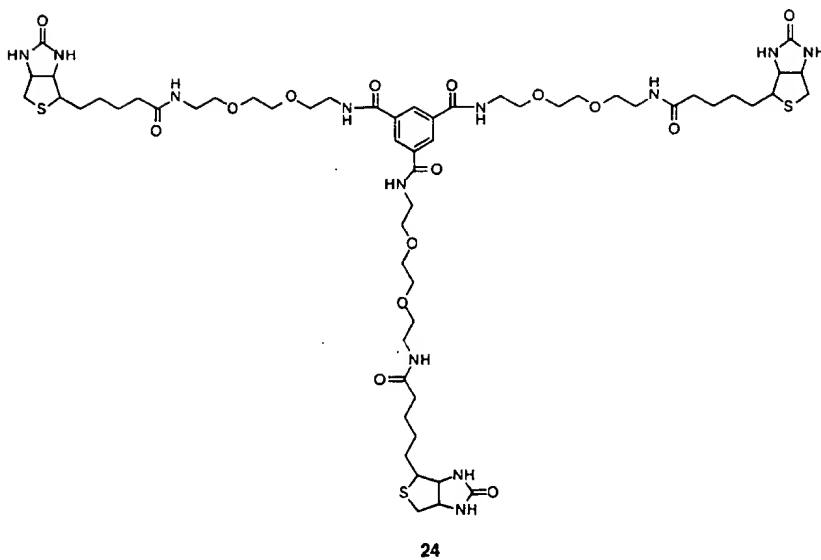
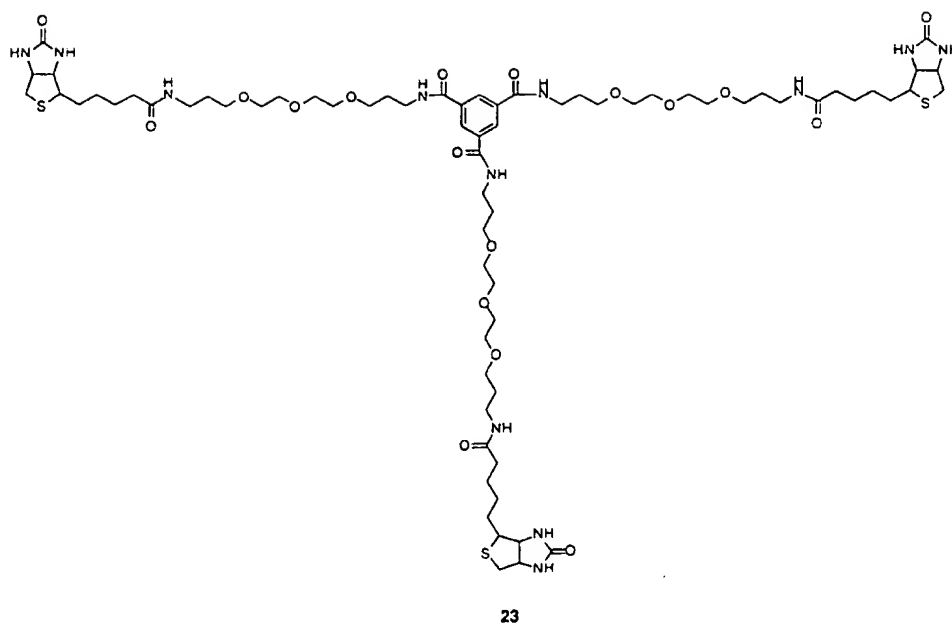
Another application of the water solubilized biotin-containing compounds according to the invention is to provide a targeting system that can be used with, for example, boron-10-containing compounds. Targeting of any number of boron-10-containing compounds, such as small molecules or polymers, to pre-selected
30 sites such as tumors can be accomplished with this system. A preferred example of a biotin boron-10-containing polymer is one prepared from starburst or cascade dendrimers (See Tomalia, in *Topics in Current Chemistry*, 165, 193-313, 1993, for a discussion of dendrimers) where a discrete number (i.e. 3-32) of biotin moieties are attached to a dendrimer and that entity is conjugated with another entity (e.g.
35 dendrimer) that contains 10-200 boron-10 cage molecules (e.g., *closo* or *nido*-borane, carbaborane, or dicarbaborane; (See Hawthorne, *Angew Chem. Int. Ed.*, Engl. 32, 950-984, 1993; Morin, *Tetrahedron* 50, 12521-12569, 1994) are

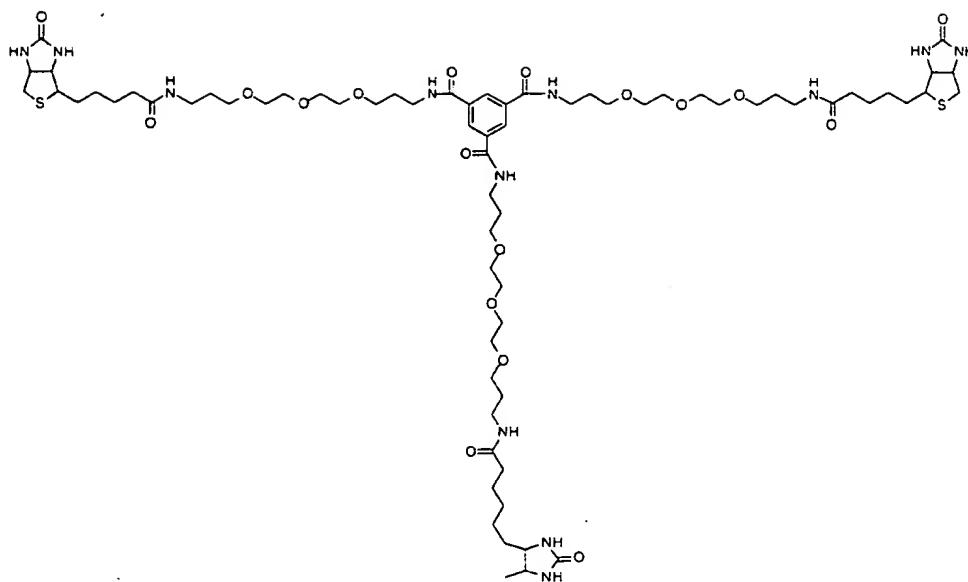
conjugated. The resulting multi-biotin-containing compound would be used with prelocalized monoclonal antibody/biotin binding proteins to localize boron-10 to selected sites such as tumors for subsequent neutron irradiation in a therapeutic protocol.

5 Trimeric, and other multimeric biotin-containing compounds in accordance with the invention can be prepared for the cross-linking of biotin-binding proteins (e.g., streptavidin and avidin). One application for polymerization of biotin-binding proteins is clearance of the antibody-biotin binding protein, or non-bound biotin-binding protein from the blood of patients which have had tumor sites targeted with
10 monoclonal antibodies conjugated with biotin-binding proteins.

One important aspect of the present invention is the determination by the inventors that the distances between any two biotin moieties on the biotin trimer of the invention be long enough ($>18\text{\AA}$) to bind at two (2) sites on the avidin/streptavidin protein, but short enough ($<60\text{\AA}$) such that the third biotin
15 moiety will not bind to the same avidin or streptavidin molecule.

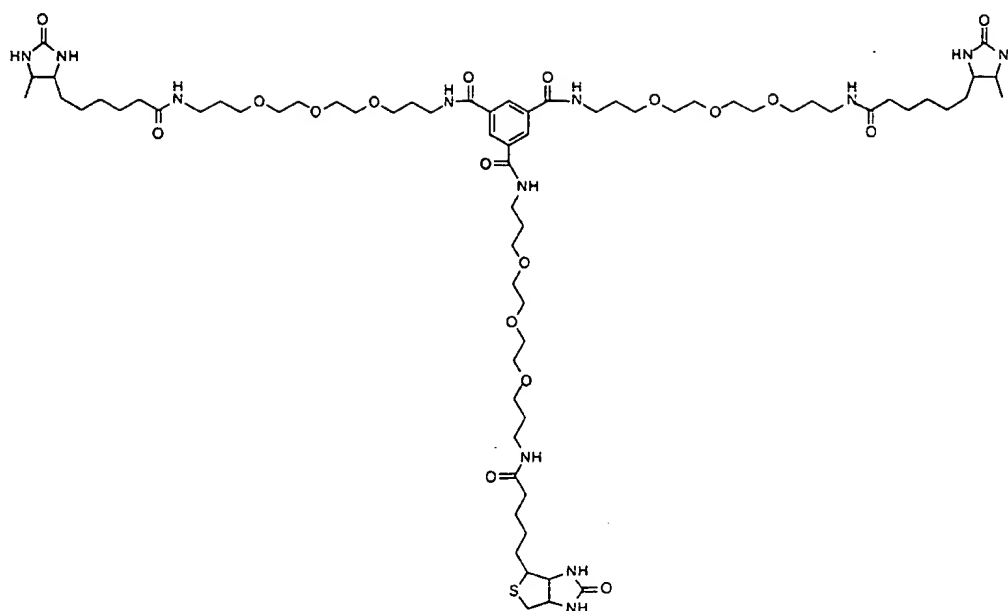
Illustrative trimeric biotin-containing compounds, in accordance with the invention, are illustrated in structures 23-26. In these compounds, the biotin moieties are a distance from one another that permits two (2) of the biotin moieties to bind with one tetrameric biotin binding molecule (e.g., avidin), while the third
20 biotin moiety will be free to bind with a separate avidin because it does not have a linker of sufficient length to bind at a third site on the same avidin molecule. The distance between each biotin moiety in the trimeric biotin-containing compound is preferably from about 20 to about 55\AA .





25

In structure **25**, one biotin moiety is the weaker binding desthiobiotin, and this can result in preparing polymers which are unstable. However, unstable
5 polymers can also be cleared from the blood, which is desirable under some circumstances. Biotin trimer **26** has two desthiobiotin moieties. In this example, four of the trimeric biotin molecules can combine with one (tetrameric) avidin or streptavidin molecule. This permits more (total of 8) desthiobiotin moieties on the molecule, and can lead to branching in the polymers formed. Biotin multimers
10 having three (3) or more biotin moieties are therefore useful for amplification of signals in diagnostic or therapeutic systems.



26

A preferred group of compounds in accordance with the invention use starburst and cascade dendrimers as the cross-linking moiety (C). For example, the reaction of the terminal amine of the starburst dendrimer (e.g., generation 2, available from commercial sources) with a biotinylation reagent, such as structure 27 (see Example 4), produces a compound which has up to 16 biotin molecules attached. Further disclosure of the dendrimers useful in the present invention are set forth below.

The multimeric biotin-containing compounds where a starburst or cascade dendrimer is used as the crosslinking agent may also comprise other functional moieties. An important application of these compounds is to increase the amount of radioactivity, photoactive moiety, or drug at a pre-selected site, such as a tumor, by introducing new biotin sites for biotin-binding proteins to bind to. Thus, methods for amplifying the number of sites for binding biotin-binding proteins at a pre-selected target, such as a tumor, would involve multiple alternating administrations of a multi-biotin-containing compound wherein the crosslinker is a starburst dendrimer and a protein, such as avidin or streptavidin. A functional reporter moiety such as a targeting, diagnostic or therapeutic agent, or the like, may be linked to the biotin-containing compound or binding protein compound. When this is accomplished in a set of steps, an amplification of the amount of sites available at the tumor is obtained, since, upon each avidin or streptavidin binding to the site, additional biotin binding sites are provided. This amplification can also be accomplished *in vitro* to improve the detection of desired samples.

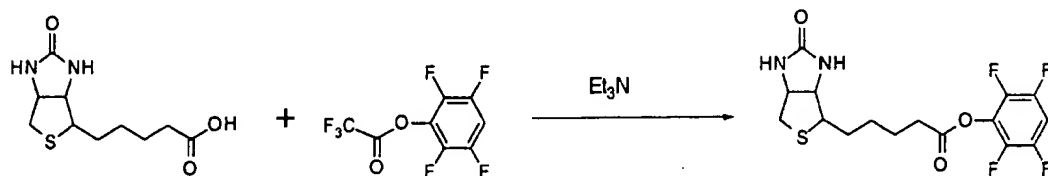
The following Examples demonstrate methods for synthesizing and using the compounds according to the invention. The Examples are provided by way of illustration, and not by way of limitation.

5 **EXAMPLE 1**

Method for Synthesizing an Activated Ester of a Biotin Moiety

The following Example sets forth a methodology for preparing a tetrafluorophenyl (TFP) ester of a biotin moiety that is subsequently reacted with a water soluble linker moiety and then a cross linker of at least tri-functionality to produce a biotin-containing compound according to the invention. Other methods for preparing activated esters generally known in the field can be used to prepare biotin activated esters containing any number of phenolic and other hydroxyl (e.g., N-hydroxysuccinimide) groups.

Preparation of biotin tetrafluorophenyl ester: Biotin (10 g, 40.9 mmol) was dissolved in 200 mL warm (70°C) DMF under an argon atmosphere. The solution was allowed to cool to ambient temperature and 10 mL (82mmol) triethylamine was added, followed by the addition of 16 g (61 mmol) 2,3,5,6-tetrafluorophenyl trifluoroacetate. The reaction was stirred at room temperature for 30 min and solvent was removed under vacuum. The product was triturated in 100 mL ether and was filtered. The reaction scheme is shown below. The isolated product was dried under vacuum to yield 14 g (83%) of biotin TFP ester as a colorless solid, mp = 185-187°C. ¹H NMR (DMSO-d₆, δ): 1.4-1.8 (m, 6H), 2.5 (m, 1H), 2.6-2.9 (m, 3H), 3.1 (m, 1H), 4.2 (m, 1H), 6.4 (d, 2H), 7.9 (m, 1H); 1R (KBr, cm⁻¹) 3250, 2915, 1790, 1710, 1520, 1520, 1480, 1090.



EXAMPLE 2

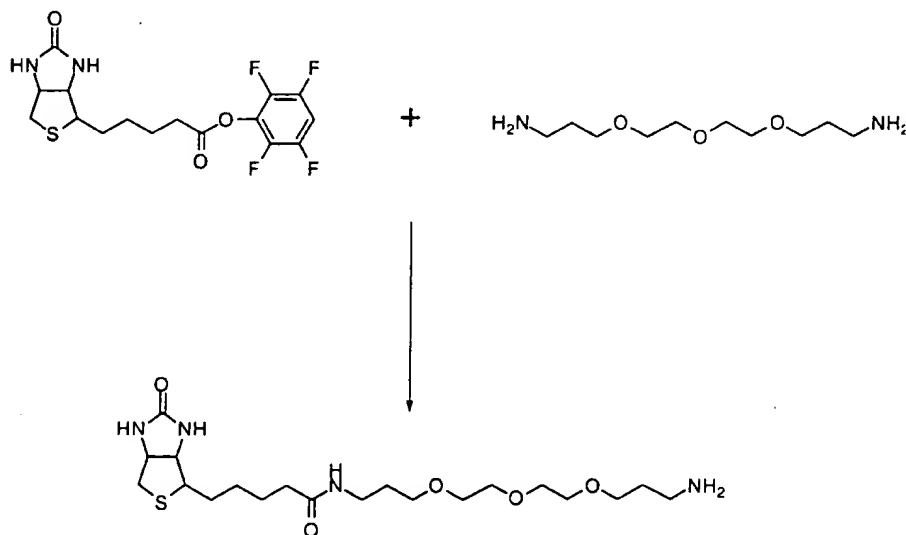
Method for Preparation of a Water Soluble Biotinylation Reagent

This Example sets forth a general methodology for preparing a biotin moiety linked to a water soluble linker moiety. In the example, a diamino-ether linker is used. Ether linkers containing terminal functionalities such as: an amine and a carboxylate; an amine and an alcohol; an alcohol and a carboxylate; and

two alcohols, can also be prepared using the method described. The general method can also be used when the linker contains polyhydroxyl groups if the terminal functionalities are two amines, or are an amine and a carboxylate.

Preparation of a biotin compound containing a trioxo-amide linker with

- 5 **an amine terminus:** The TFP ester of biotin (5 g, 12.8 mmol) (Example 1) was dissolved in 200 mL anhydrous DMF. In another flask containing 28 g (128 mmol) 4,7,10-trioxa-1,13-tridecanediamine was added 4 mL of triethylamine. Both the flasks were cooled to 0-5°C by ice water. The TFP ester of biotin was added, dropwise, to the trioxatridecanediamine solution over the period of 1 hour. The mixture was stirred at room temperature for 30 minutes and the solvent was removed under vacuum. The resulting oil was triturated in 500 mL ether and was stirred for 30 minutes. The solid was filtered. The solid product was dissolved in methanol:ethyl acetate (8:2) and loaded on silica column. The column was eluted with methanol:ethyl acetate (8:2). Fractions containing product were collected and the solvent was removed under vacuum. The isolated product was dried under vacuum to yield 4.5 g (79%) of desired product as a colorless solid, mp = 104-106°C. ¹H NMR (MeOH, δ): 1.46 (m, 2H), 1.6-1.8 (m, 9H), 2.2 (t, 2H), 2.7 (d, 1H), 2.75-2.9 (m, 3H), 3.2-3.3 (m, 5H), 3.5-3.6 (m, 14H); 4.3 (m, 1H), 4.5 (m, 1H), 1R (KBr, cm⁻¹) 3280, 2910, 2850, 1690, 1640, 1110, 940.

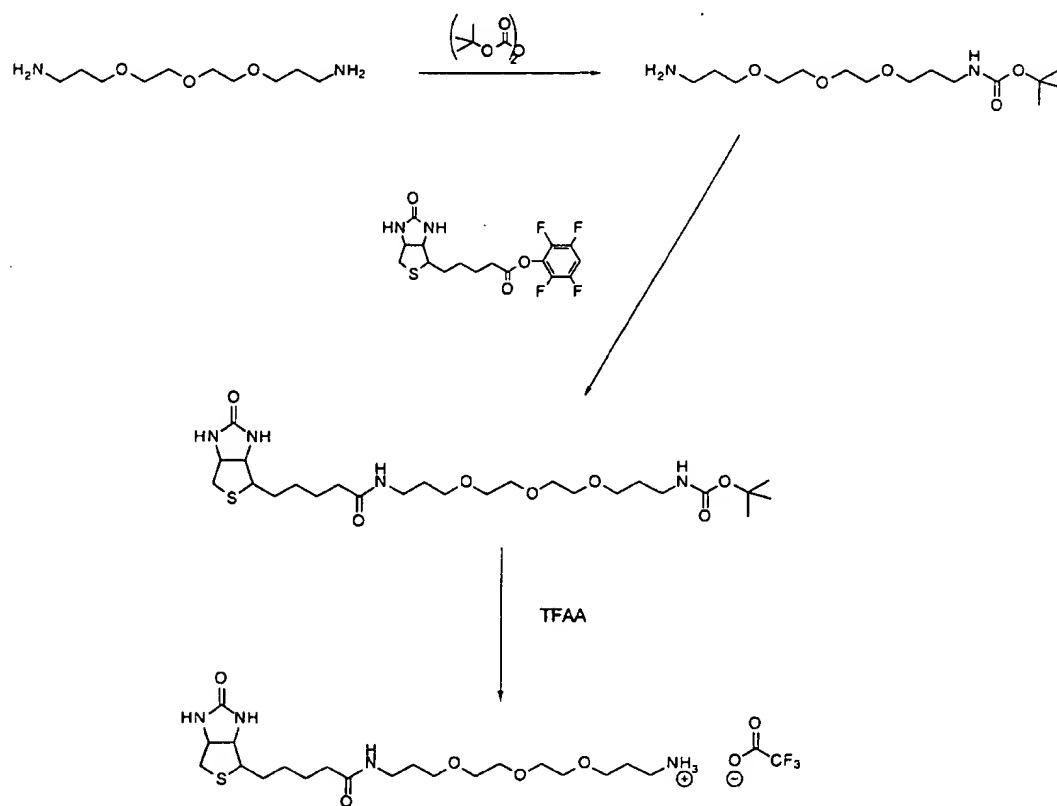


20

EXAMPLE 3

Reacting an Activated Ester of Biotin with a Diamino-linker that has One Amine Protected

This Example sets forth a general methodology for preparing a biotin/water soluble linker adduct where the water soluble linker has one amine group protected. In the example, a diamino-ether linker was protected as a *t*-Boc derivative prior to addition to the biotin activated ester. Any number of amine protecting groups can be utilized in this reaction. The primary purpose of the reaction method as opposed to Example 2, is to be able to react one equivalent of diamino linker molecule with one equivalent of the biotin active ester. The reaction scheme is set forth below.



Reaction Step 1: Preparation of N-Boc-4,7,10-trioxatridecane-13-amine:

To a solution of 151.40 g (687.25 mmol) of 4,7,10-trioxa-1,13-tridecaneamine in 700 mL CHCl_3 was added 6.00 g (27.5 mmol) of di-*tert*-butyl dicarbonate in 100 mL CHCl_3 with stirring at ambient temperature over 30 minutes. The mixture was stirred for 12 hours, washed with water (8 x 100 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 8.23 g (25.7 mmol) of the desired product as a clear oil: ^1H NMR (CDCl_3 , δ): 5.18 (s, 1H, CONH), 3.55 (m,

12H $-OCH_2$), 3.20 (m, 2H CH_2NHCO), 2.68 (t, 2H CH_2NH_2), 1.73 (m, 4H), 1.41 (s, 9H).

Reaction Step 2: Preparation of N-(13-N-Boc-4,7,10-trioxatridecanyl)biotinamide:

To a solution containing 1.00 g (2.54 mmol) 2,3,5,6-tetrafluorophenol ester of biotin in 100 mL CH_3CN at 55°C was added 0.80 g (2.49 mmol) N-Boc-4,7,10-trioxa-13-tridecaneamine in 25 mL CH_3CN . The mixture was allowed to cool to ambient temperature and was stirred for 10 hours. The solvent was evaporated under reduced pressure and the residue was re-dissolved in 200 mL EtOAc. The EtOAc solution was washed with (2 x 100 mL) 10% solution of $NaHCO_3$, (2 x 50 mL) water, dried over anhydrous Na_2SO_4 and concentrated to give the desired product as a tacky solid. 1H NMR ($CDCl_3$, δ): 6.71 (s, NH, 1H), 6.53 (s, NH, 1H), 5.89 (s, NH, 1H), 5.06 (s, NH, 1H), 4.47 (m, 1H), 4.27 (m, 1H), 3.57 (m, 12H), 3.30 (m, 2H), 3.17 (m, 2H), 3.11 (m, 1H), 2.87 (dd, $J = 12.8$, 4.5 Hz, 1H), 2.71 (d, $J = 12.8$ Hz, 1H), 2.15 (t, 2H), 1.72 (m, 10H), 1.40 (s, 9H).

Reaction Step 3: Preparation of 13-N-Biotinamide-4,7,10-trioxatridecaneamine trifluoroacetic acid salt: N-Boc-trioxa-biotinamide, 1.25 g (2.28 mmol), was dissolved in 10 mL trifluoroacetic acid and stirred for 30 min at ambient temperature. Excess trifluoroacetic acid was evaporated under reduced pressure to give 1.24 g (2.17 mmol) of the desired trifluoroacetate salt as an oil: 1H NMR (CD_3OD , δ): 4.25 (m, 1H), 4.13 (m, 1H), 3.35 (m, 10H), 3.32 (m, 2H), 2.97 (m, 4H), 2.81 (m, 2H), 2.65 (dd, $J = 12.8$, 4.8 Hz), 2.63 (d, $J = 12.8$ Hz), 1.93 (m, 2H), 1.64 (m, 2H), 1.48 (m, 4H), 1.37 (m, 2H), 1.18 (m, 2H).

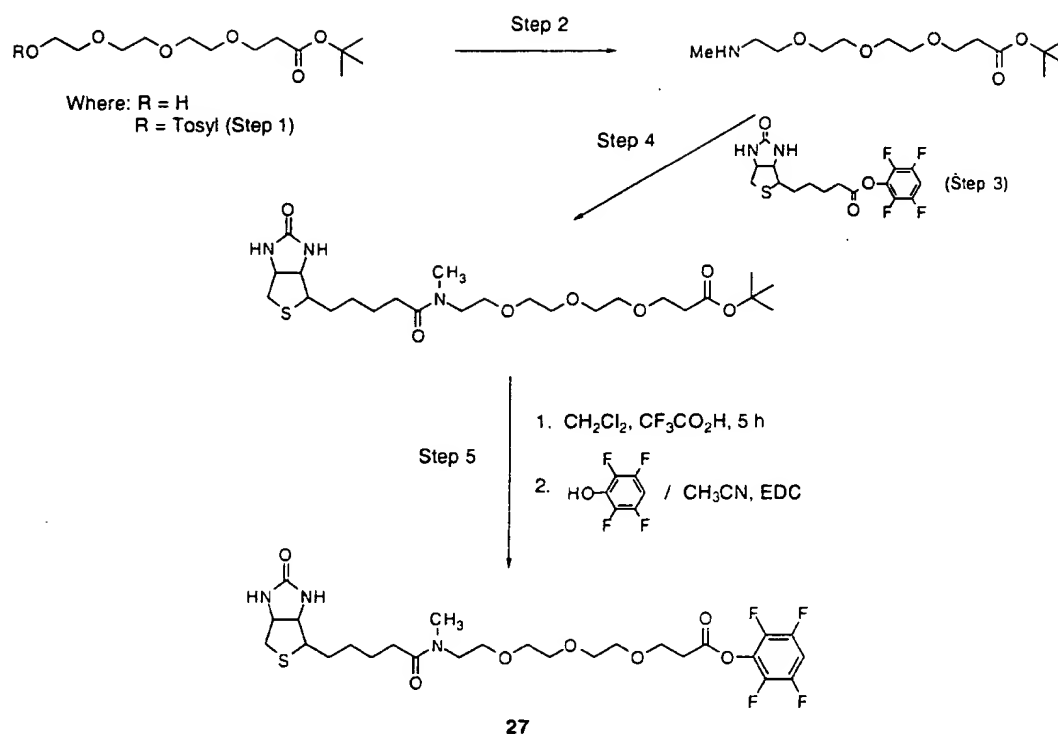
EXAMPLE 4

Method of Preparing a Carboxy Derivative of a Water Soluble,

Biotinidase-Stabilized Biotinylation Reagent

This Example sets forth a general methodology for preparing a biotin/water soluble linker adduct comprising an ω terminal carboxylate. The method described can be applied in a general way to prepare various biotin/water soluble linker adducts. While in the reaction described, an amine and ester terminated ether linker is used, reagents wherein the linker has other combinations of amines, carboxylates, thiols, and alcohols at the termini are also provided for with this method.

The reaction scheme for this Example is set forth below.



Reaction Step 1: 13-O-*p*-Tosyl-4,7,10,13-tetraoxadodecanoic Acid *tert*-Butyl Ester .

To a solution of 12-hydroxy-4,7,10-trioxadodecanoic acid *tert*-butyl ester (15 g, 54 mmol) in 100 mL pyridine at 0-5°C was added *p*-toluenesulfonyl chloride (20.6 g, 108 mmol). The reaction mixture was stirred at 0°C for 4 hours, poured into ice - H₂O and the aqueous layer was extracted with CH₂Cl₂ (3 x 125 mL). The combined organic layers were washed with 2% HOAc and H₂O and then dried over anhydrous Na₂SO₄. The solvent was removed under vacuum to yield a colorless oil. The oil was dried under vacuum to give 22 g (96%) of the desired product. ¹H NMR (CDCl₃) δ 1.42 (s, 9H), 2.48 (s, 3H), 2.5 (t, 2H), 3.5-3.8 (m, 13H), 4.2 (m, 2H), 7.3 (d, 2H), 7.8 (d, 2H). IR (Nujol, cm⁻¹) 3420, 1720, 1630, 1590, 1450, 1360, 1250, 920. HRMS calculated for C₂₀H₃₂O₈SNa (M+Na) is 455.1716; found 455.1707.

Reaction Step 2: 12-N-Methylamino-4,7,10-trioxadodecanoic Acid *tert*-Butyl Ester.

To a solution of 13-O-*p*-tosyl-4,7,10,13-tetradecanoic acid *tert*-butyl ester (11 g, 25.3 mmol) in THF (10 mL) was added a 2 M solution of methylamine in THF (176 mL, 352 mmol). The reaction was stirred at room temperature for 40 hours. The solvent was removed under the vacuum. The residue was extracted with chloroform (3 x 100 mL). The combined chloroform extracts were washed with (2 x 50 mL) H₂O, dried over anhydrous Na₂SO₄, and the CHCl₃ was removed

under vacuum. The product was loaded on a silica column. The column was first eluted with ethyl acetate, and then with varying percentages of ethyl acetate : methanol until 100% MeOH was eluted. The fractions containing product were reduced in volume and the isolated product was dried under vacuum to yield 3 g (40%) of the desired product as an oil. ¹H NMR (CDCl₃) δ 1.4 (s, 9H), 2.38 (s, 3H), 2.42 (t, 2H), 2.7 (m, 2H), 3.5-3.8 (m, 13H). IR Nujol, cm⁻¹) 3320, 1720, 1550, 1450, 1360, 1250, 1100, 940. HRMS calculated for C₁₄H₃₀NO₅ (M+H)⁺ is 292.2124; found 292.2116.

Reaction Step 3: Biotin tetrafluorophenyl ester. This compound was prepared by reaction of biotin with 2,3,5, 6-tetrafluorophenyl trifluoroacetate as described in Example 1.

Reaction Step 4: 12-N-Biotinyl-(N-methyl)amino-4,7,10-trioxadodecanoic Acid *tert*-Butyl Ester. To a solution of 12-N-methylamino-4,7,10-trioxadodecanoic acid *tert*-butyl ester (0.12 g, 0.412 mmol) in 25 mL of DMF was added Et₃N (60 μL, 0.412 mmol), followed by biotin TFP ester (0.161 g, 0.412 mmol). The reaction was stirred at room temperature for four (4) hours and the solvent was removed under vacuum. The residue was extracted with CHCl₃ (300 mL). The CHCl₃ solution was washed with saturated aqueous NaHCO₃ (3 x 25 mL), then H₂O (2 x 25 mL) and was dried over anhydrous Na₂SO₄. The CHCl₃ was removed under vacuum to yield 0.21 g (98%) of the product as an oil. ¹H NMR (CDCl₃) δ 1.4 (s, 10H), 1.6-1.8 (m, 4H), 2.3 (t, J = 4.25 Hz, 3.9 Hz, 1H), 2.4 (t, J = 3.75 Hz, 3.6 Hz, 1H), 2.5 (t, J = 3.5 Hz, 3.4 Hz, 2H), 2.8 (d, J = 6.75 Hz, 1H), 2.9-3.0 (m, 4H), 3.1 (s, 2H), 3.2 (bs, 1H), 3.45-3.7 (m, 13H), 4.35 (s, 1H), 4.55 (s, 1H), 5.7 (d, J = 7.5 Hz, 1H), 6.1 (d, J = 10.5 Hz, 1H). IR Nujol, cm⁻¹) 3260, 3060, 1780, 1690, 1520, 1480, 1260, 1080, 930. HRMS calculated for C₂₄H₄₄N₃O₇S (M+H)⁺ is 518.2900; found 518.2923.

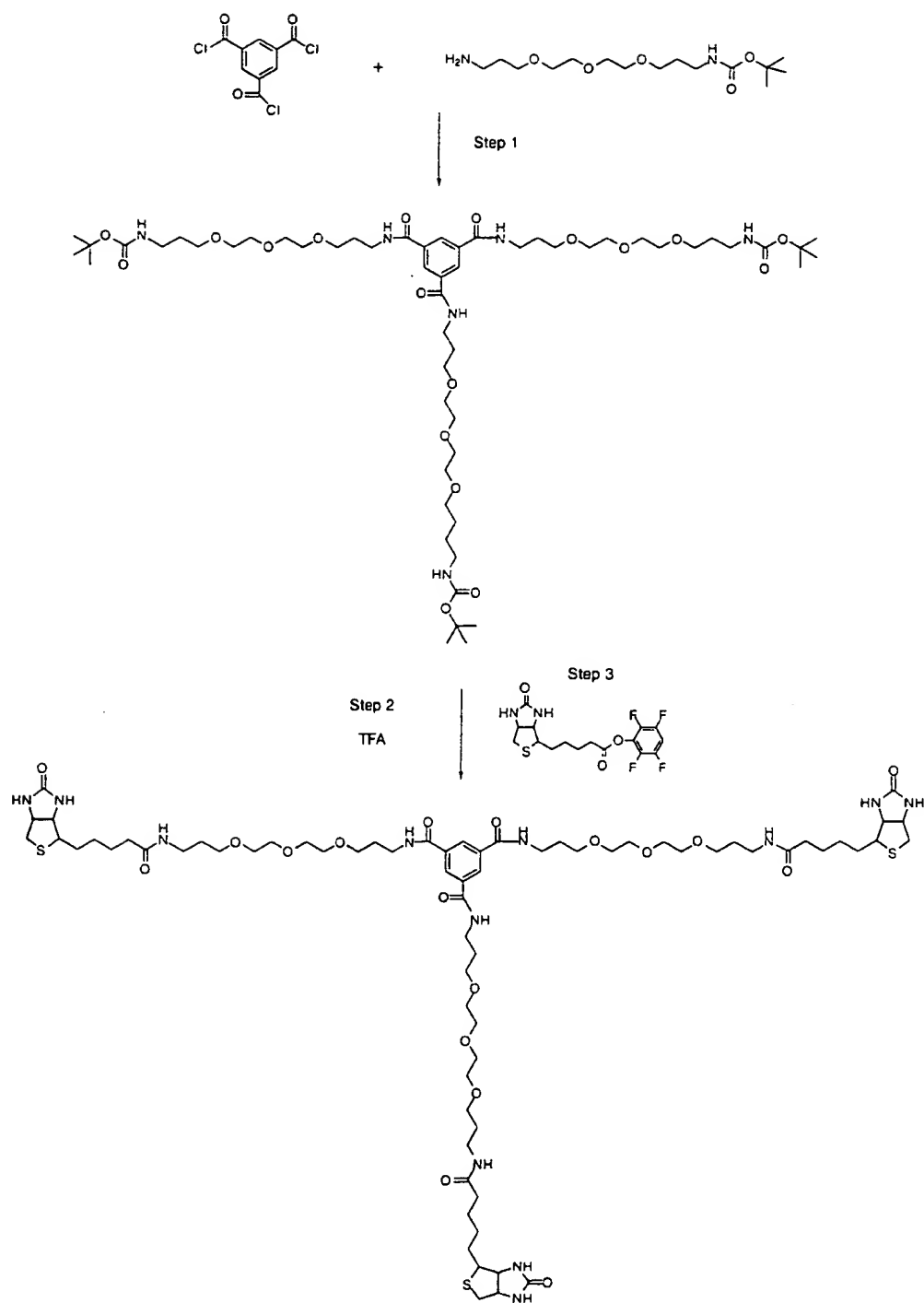
Reaction Step 5: 12-N-Biotinyl-(N-methyl)amino-4,7,10-trioxadodecanoic Acid Tetrafluorophenyl Ester. To a solution of 12-N-biotinyl-(N-methyl)amino-4,7,10-trioxadodecanoic acid *tert*-butyl ester (0.21 g, 0.406 mmol) in 10 mL CH₂Cl₂ was added CF₃CO₂H (1 mL). This mixture was stirred at room temperature for 5 hours. The mixture was reduced in volume under vacuum and the residue acid intermediate was dissolved in 20 mL of CH₃CN. Tetrafluorophenol (0.083 g, 0.5 mmol) was added, followed by EDC (0.096 g, 0.5 mmol). The reaction mixture was stirred at room temperature for 16 hours and the solvent was removed under vacuum. The residue was extracted with CHCl₃ (300 mL), washed with saturated aqueous NaHCO₃ (3 x 25 mL), then H₂O (2 x 25 mL) and dried over anhydrous

Na₂SO₄. The solvent was removed under vacuum to yield 0.243 g (93%) of the product as an oil. ¹H NMR (CDCl₃) δ 1.4 (s, 2H), 1.5-1.7 (m, 4H), 2.3 (m, 2H), 2.65 (d, J = 6.25 Hz, 1H), 2.8-2.9 (m, 4H), 3.0 (s, 2H), 3.4-3.6 (m, 13H), 3.8 (s, 2H), 4.35 (s, 1H), 4.45 (s, 1H), 6.0 (d, J = 8.25 Hz, 1H), 6.3 (d, J = 6.75 Hz, 1H), 6.95 (m, 1H).
5 IR Nujol, cm⁻¹) 3260, 3060, 1780, 1690, 1520, 1480, 1260, 1080, 930. HRMS calculated for C₂₆H₃₅N₃O₇F₄S (M+H)⁺ is 610.2210; found 610.2216.

EXAMPLE 5

Method for Preparing a Multi-Biotin-Containing-Compound with a 35

10 Atom Distance Between the Biotin Carboxylates



Reaction Step 1, Preparation of N,N',N''-tris-(13-N-Boc-4,7,10-trioxatridecanyl) benzene-1,3,5-tricarboxamide: To a solution of benzene-1,3,5-tricarbonyl trichloride (0.28 g, 1.05 mmol) in 10 mL of CH_2Cl_2 at 0°C was added 0.96 g (2.99 mmol) of 13-N-Boc-4,7,10-trioxa-tridecaneamine (see Example

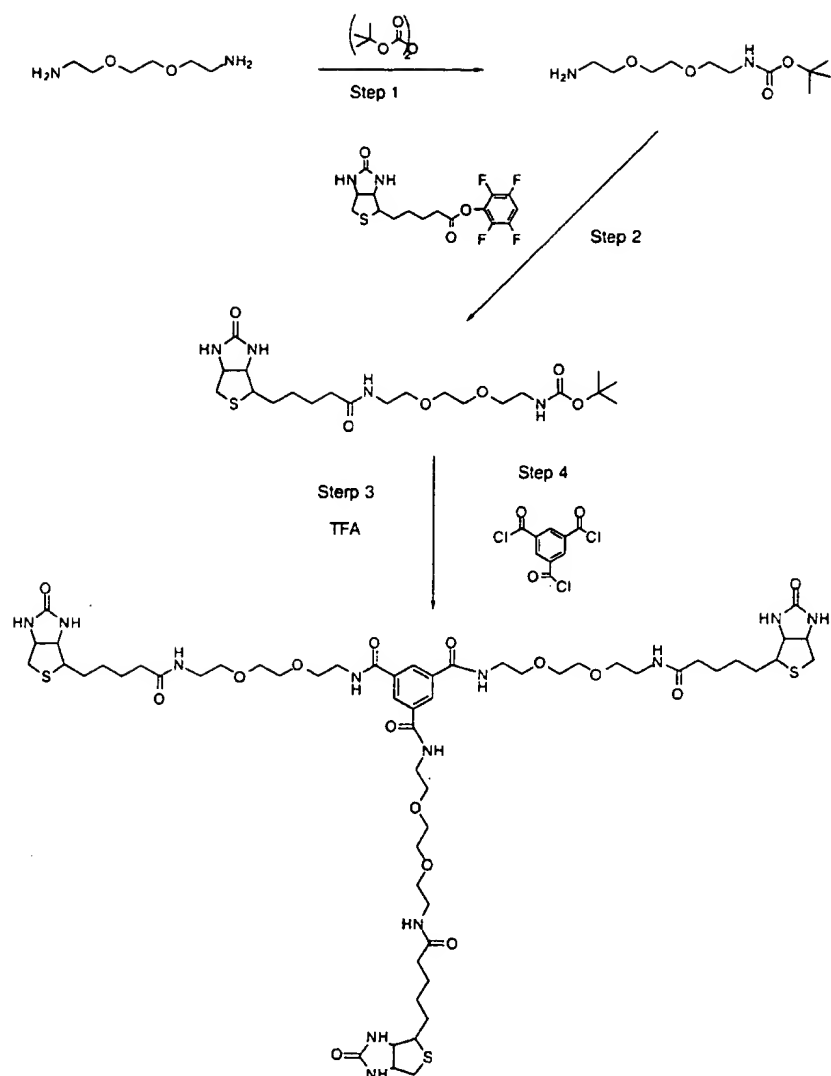
3) and 1.45 g (14.34 mmol) triethylamine in 5 mL CH₂Cl₂. That mixture was stirred under argon for 10 hours at ambient temperature and the volatile materials were removed under reduced pressure. The residue was redissolved in 150 mL CH₂Cl₂, washed with 20 mL of water, dried over anhydrous Na₂SO₄ and evaporated to give 0.94 g (83%) the desired biotin trimer as a tacky solid. ¹H NMR (CDCl₃, δ) 8.38 (s, 3H), 7.64 (s, ArCONH, 3H), 5.16 (s, Boc-NH, 3H), 3.62 (m, 30H), 3.55 (m, 6H), 3.46 (m, 6H), 1.89 (m, 6H), 1.67 (m, 6H), 1.41 (s, 27H).

Reaction Step 2: N,N',N''-tris-(13-amino-4,7,10-trioxatridecanyl)benzene-1,3,5-tricarboxamide trifluoroacetic acid salt: The N-Boc protected triamide (0.25 g, 0.23 mmol) was dissolved in 5 mL trifluoroacetic acid at ambient temperature and stirred for 30 minutes. Excess trifluoroacetic acid was evaporated under reduced pressure to give 0.26 g (0.22 mmol) of the trifluoroacetic acid salt as an oil. ¹H NMR (CD₃OD) δ 8.47 (s, 3H), 3.64 (m, 36H), 3.60 (m, 6H), 3.14 (m, 6H), 1.94 (m, 12H).

Reaction Step 3: N,N',N''-tris(13-N-Biotinamide-4,7,10-trioxatridecanyl)benzene-1,3,5-tricarboxamide: To 0.19 g (0.18 mmol) of the tris trifluoroacetate salt in 3 mL DMF was added 1 mL (7.17 mmol) triethylamine, followed by 0.24 g (0.61 mmol) of biotin tetrafluorophenol ester in 2 mL DMF. The solvents were evaporated under reduced pressure and the residue was washed with 2 mL water, 2 mL of a 10% solution of NaHCO₃, 2 mL water, then 2 mL acetone. The residue was crystallized from methanol/acetone to give 0.04 g (11%) as colorless crystals. mp = 106-108°C; ¹H NMR (CD₃OD, δ) 8.44 (s, 3H), 4.49 (m, 3H), 4.31 (m, 3H), 3.60 (m, 36H), 3.23 (m, 12H), 2.91 (m, 3H), 2.71 (d, J = 12.7 Hz), 2.15 (m, 12H), 1.91 (m, 6H), 1.65 (m, 12H), 1.40 (m, 6H), MS (FAB⁺): mass calculated for C₆₉H₁₁₄N₁₂O₁₆S₃ is 1495; found *m/z* isotope cluster at 1496-1499 (M+H).

EXAMPLE 6

Method for Preparing a Water Soluble Biotin Trimer with a 25 Atom Distance Between the Biotin Carboxylates



Reaction Step 1: Preparation of 8-N-Boc-3,6-dioxaoctanamine: To a solution of 163 g (1100 mmol) of 3,6-dioxo-1,8-octanediamine in 700 mL CHCl_3 was added 8.00 g (36.65 mmol) of di-*tert*-butyl dicarbonate in 100 mL CHCl_3 with stirring at ambient temperature over 30 minutes. The mixture was stirred for 12 hours, washed with (8 x 100 mL) water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 8.62 g (85%) of the desired product as a colorless oil. ^1H NMR (CDCl_3 , δ) 5.40 (s, 1H, CONH), 3.63 (m, 4H, $-\text{OCH}_2$), 3.54 (m, 4H, OCH_2), 3.32 (m, 2H, CH_2NHCO), 2.87 (t, 2H, CH_2NH_2), 1.46 (s, 9H), 1.40 (s, 1H, NH_2).

Reaction Step 2, Preparation of N-(8-N-Boc-3,6-dioxaoctanyl)biotinamide: To a solution containing 1.00 g (2.54 mmol) 2,3,5,6-tetrafluorophenol ester of biotin in 100 mL CH_3CN at 55°C was added 0.62 g (2.50 mmol) N-Boc-4,7,10,13-trioxa-

13-tridecaneamine in 20 mL CH₃CN. The mixture was allowed to cool to ambient temperature, and was stirred for 8 hours. The solvent was evaporated under reduced pressure and the residue was redissolved in 150 mL EtOAc. The EtOAc solution was washed with (2 x 75 mL) 10% aqueous NaHCO₃ and (2 x 75 mL) water, dried over anhydrous Na₂SO₄ and concentrated to give 1.03 g (87%) as a colorless solid. mp = 99-101°C; ¹H NMR (CDCl₃) δ 6.83 (s, NH, 2H), 6.05 (s, NH, 1H), 5.21 (s, NH, 1H), 4.48 (m, 1H), 4.29 (m, 1H), 3.60 (m, 4H), 3.53 (m, 4H), 3.43 (m, 2H), 3.29 (m, 2H), 3.12 (m, 1H), 2.88 (dd, J = 12.8, 4.8 Hz, 1H), 2.73 (d, J = 12.8 Hz, 1H), 2.21 (t, 2H), 1.68 (m, 4H), 1.43 (s, 11, t-Butyl and CH₂).

- 10 **Reaction Step 3, Preparation of 8-N-Biotinamide-3,6-dioxaoctaneamine trifluoroacetic acid salt:** The N-Boc-trioxabiotinamide (1.03 g, 2.17 mmol) was dissolved in 10 mL trifluoroacetic acid and stirred for 30 minutes at ambient temperature. Excess trifluoroacetic acid was evaporated under reduced pressure to give 0.96 g (90%) of the desired product as an oil. ¹H NMR (CD₃OD) δ 4.10 (m, 1H), 3.90 (m, 1H), 3.28 (m, 4H), 3.24 (m, 4H), 3.14 (m, 2H), 2.95 (m, 2H), 2.79 (m, 1H), 2.51 (dd, J = 12.8, 4.8 Hz), 2.29 (d, J = 12.8 Hz), 1.81 (t, 2H), 1.25 (m, 4H), 1.02 (m, 2H).

- Reaction Step 4, Preparation of N,N',N''-tris(8-N-Boc-3,6-dioxaoctanyl)benzene-1,3,5-tricarboxamide:** To a solution containing 0.28 g (1.05 mmol) of benzene-1,3,5-tricarbonyl trichloride in 10 mL CH₂Cl₂ at 0°C was added 0.74 g (2.98 mmol) 8-N-Boc-3,6-dioxaoctaneamine and 0.70 g (6.91 mmol) triethylamine in 5 mL CH₂Cl₂. The reaction mixture was stirred under argon for 10 hours at ambient temperature and the volatile material was evaporated under reduced pressure. The residue was redissolved in 150 mL CH₂Cl₂, washed with 20 mL water, dried over anhydrous Na₂SO₄ and evaporated to give 0.82 g (87%) of the desired product as a tacky solid. ¹H NMR (CDCl₃) δ 8.33 (s, 3H), 7.67 (s, ArCONH, 3H), 5.45 (s, Boc-NH, 3H), 3.65 (m, 24H), 3.53 (m, 6H), 3.26 (m, 6H), 1.39 (s, 27H).

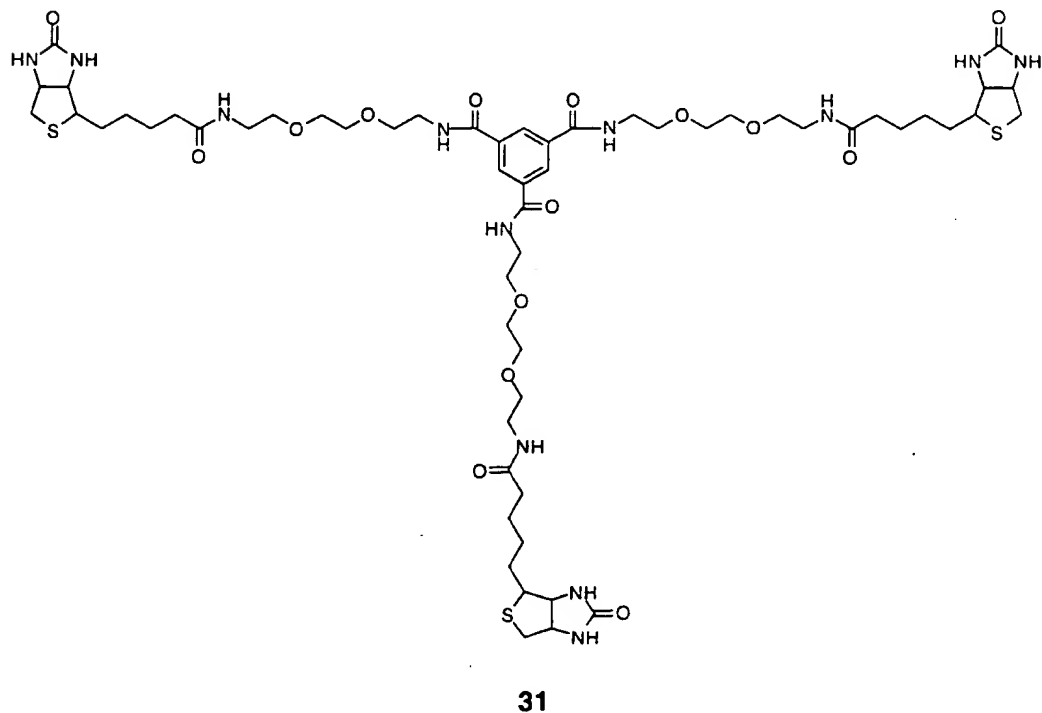
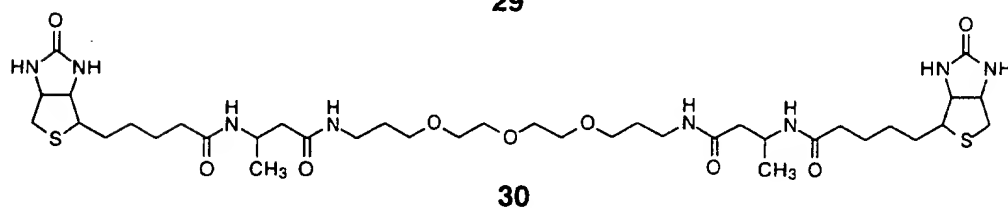
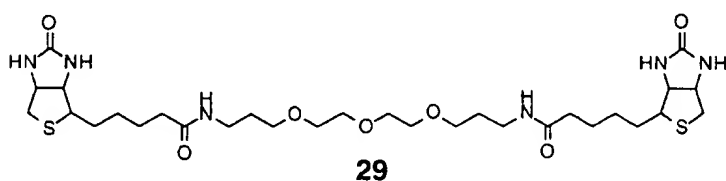
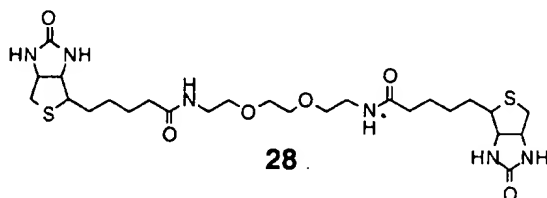
30 **EXAMPLE 7**

Streptavidin Cross-Linking with Biotin Compounds

- This experiment was designed to test streptavidin cross-linking with biotin monomer, biotin dimers comprising water soluble linkers, and biotin moiety trimers comprising water soluble linkers. Each of the biotin dimers and trimers used experimentally was water soluble, that is, each biotin compound had a water

solubility in excess of 0.2 mg/mL at neutral pH and ambient temperature. The Biotin dimers used herein are structures **28**, **29** and **30**. The trimers used herein are structures **23** (page 26), **24** (page 26) and **31**. The biotin monomer used herein is the N-[13-(*p*-iodobenzamido)-4,7,10-trioxatridacanyl]biotinamide.

5



A Reacti-Bind Streptavidin Coated Polystyrene Strip Place (Pierce) was washed with 100 mM PBS prior to use. To lane one (12-wells) was added 100 μ L

PBS. To lanes 2-7 were added 100 pmol of biotin dimer or biotin trimer to be tested in 100 μ L PBS. Lane 8 was treated in the same manner with 100 pmol of biotin monomer. The plate was then incubated at 37°C with shaking for 10 minutes. All the wells were then emptied and rinsed with 100 μ L PBS. The PBS was removed and 25 pmol of 125 I labeled streptavidin (specific activity of 1 μ Ci/ μ g) was added to each well in 100 μ L PBS. The plate was then incubated with shaking for another 10 minutes. The streptavidin was removed and all the wells were washed with 100 μ L PBS. After removal of the PBS, the first 3 wells of each lane were again filled with 100 μ L PBS. The remaining wells were then filled with 100 μ L of their respective biotin dimer or biotin trimer as in the first step. The plate was then incubated again for 10 minutes and all wells were rinsed with PBS as before. Another 100 μ L of the radiolabeled streptavidin was then added to all of the wells, 100 μ L PBS was added to the first 6 wells of each lane. The remaining 6 wells in each lane were filled with 100 μ L of their respective dimer or trimer and again incubated for 10 minutes at 37°C. After removing the liquid in all the wells and washing with PBS, 100 μ L of the radiolabeled streptavidin was again added to each well followed by incubation. The streptavidin was removed and the wells washed with PBS. The first 9 wells in each lane were then filled with 100 μ L PBS and the remaining 3 wells in each lane were filled with 100 μ L of their respective dimer or trimer. After incubating and washing, 100 μ L of radiolabeled streptavidin was again added to each well. After incubating for 10 minutes, each well was washed with 100 μ L of PBS and all liquid removed. The wells were then separated and placed in a gamma counter. The amount of activity remaining in each well was determined as a percentage of the highest initial streptavidin binding.

Figure 1 illustrates the percentage streptavidin binding of the biotin monomer and the dimers and trimers as a function of sequential biotin compound additions. Each of the biotin trimers used demonstrated increased streptavidin binding with each sequential biotin trimer addition. Neither the biotin monomer nor any of the biotin dimers tested demonstrated appreciable increases in streptavidin binding with sequential biotin compound additions.

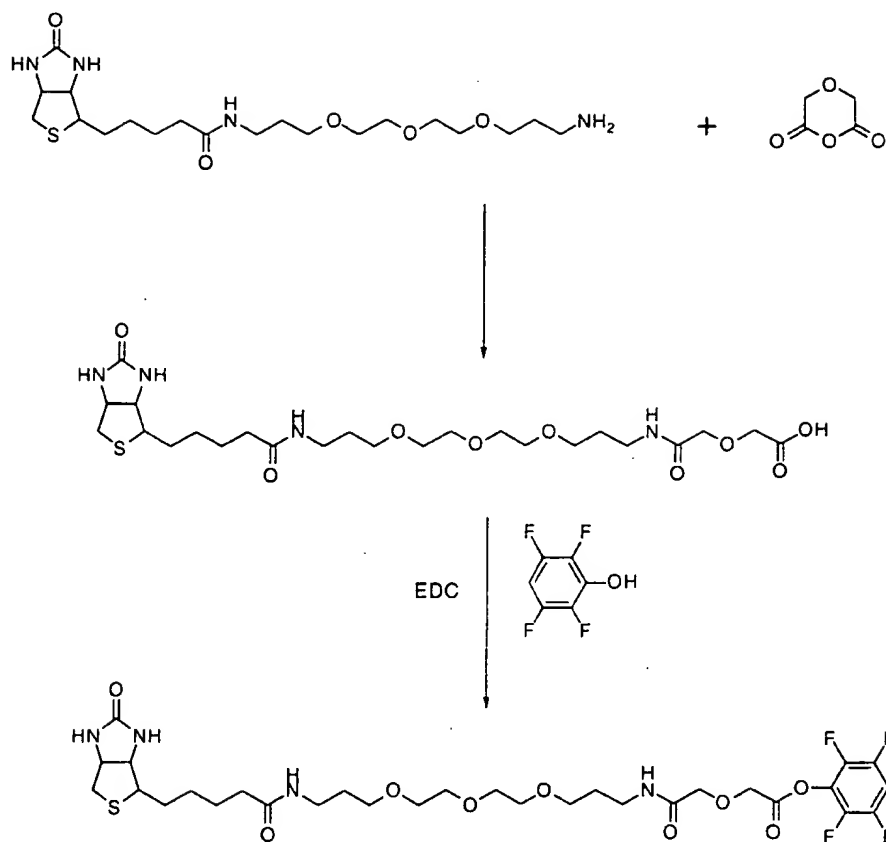
This experimental data demonstrates that biotin trimers of the present invention can be successfully used to provide amplification of binding sites for complimentary binding moieties, such as streptavidin. The water solubility of such biotin trimers and multimers is particularly important for *in vivo* applications.

The following examples set forth a general methodology for preparing the biotin/water soluble reagents which are then reacted with a cross linker of at least tri-functionality. For example, the TFP ester is prepared by esterification of the biotin-trioxaamido-glycolate with tetrafluorophenol. Under the same reaction
5 conditions, any number of activated esters can be prepared by substitution of different phenols, or other alcohols (e.g., N-hydroxysuccinimide).

EXAMPLE 8

10 Method for Preparing a Water Soluble Biotinylation Reagent that is Reactive with Amines

Biotin-4,7,10-trioxa-1,13-tridecanediamine (1 g, 2.24 mmol) was added to a dry flask and was dissolved in anhydrous $\text{CH}_3\text{CN}/\text{DMF}$ (100/25 mL). To this
15 solution was added 0.4 mL (2.9 mmol) triethylamine, followed by 0.31 g (2.7 mmol) diglycolic anhydride. The reaction was stirred at room temperature for 1 hour, and the solvent was removed under vacuum. The product was triturated in 100 mL ether and was filtered. The resultant filtrate was dried under vacuum to yield 1.26 g (99%) of desired product as a colorless solid, mp 117-119°C; ^1H NMR
20 (CDCl_3 , δ): 1.3-1.7 (m, 10H), 2.1 (t, 2H), 2.7 (d, 1H), 2.8 (dd, 1H), 3.1 (m, 1H), 3.2-3.3 (m, 4H), 3.4-3.55 (m, 13H); 3.99 (s, 2H), 4.07 (s, 2H), 4.28 (m, 1H), 4.43 (m, 1H), 5.9 (s, 1H), 6.8 (m, 2H), 7.4 (t, 1H).



The biotin - 4,7,10-trioxa-1,13-tridecanediamine – diglycolic carboxylate
 5 (1.0 g, 1.78 mmol) thus prepared, was then placed in a dry flask and was
 dissolved in anhydrous $\text{CH}_3\text{CN}/\text{DMF}$ (75/25 mL). Tetrafluorophenol (2.13 mmol,
 0.353 g) was added to the solution followed by 0.41 g (2.13 mmol) EDC. The
 reaction was stirred at room temperature for 1 hour (followed by HPLC), and the
 solvents were removed under vacuum. The residue was extracted with 200 mL
 10 chloroform, washed with (2 x 25 mL) saturated sodium bicarbonate solution, then
 with (2 x 50 mL) water. The chloroform solution was dried over anhydrous
 sodium sulfate and the chloroform was removed under vacuum. The product
 was triturated in 100 mL ether and was filtered. The filtrate was dried under
 vacuum to yield 0.8 g (63%) of desired product as a solid. ^1H NMR (CDCl_3 , δ) 1.3-
 15 1.8 (m, 10H), 2.1 (t, 2H), 2.7 (d, 1H), 2.8 (dd, 1H), 3.1 (m, 1H), 3.2-3.6 (m, 15H), 3.8
 (t, 2H), 4.08 (s, 1H), 4.27 (m, 3H), 4.4 (m, 2H), 5.7 (s, 1H), 6.4 (s, 1H), 6.5 (m, 1H),
 7.0 (m, 1H).

EXAMPLE 9**Method for Preparing a Water Soluble Biotinylation Reagent that is Reactive****5 with Sulfhydryls or Amines**

Biotin-4,7,10-trioxa-1,13-tridecanediamine (1.0 g, 2.2 mmol) was dissolved in 12 mL of saturated aqueous sodium bicarbonate and was cooled with ice water.

- 10 N-methoxycarbonyl-maleimide (4.5 mmol, 0.696 g) was added and the reaction was stirred at 0°C for 10 minutes. A 50 mL quantity of water was added to the reaction and the stirring was continued at room temperature for an additional 15 minutes. The solution was extracted with (4 x 100 mL) chloroform. The combined chloroform extracts were washed with (2 x 50 mL) water, dried over anhydrous sodium sulfate, and chloroform was removed under vacuum. The product was
- 15 triturated in 100 mL ether and was filtered. The isolated product was dried under vacuum to yield 0.57 g (49%) of the compound 18 as a colorless solid; mp = 112-114°C; ¹H NMR (MeOH, δ): 1.46 (m, 2H), 1.6-1.8 (m, 9H), 2.2 (t, 2H), 2.7 (d, 1H), 2.9 (dd, 1H), 3.2-3.3 (m, 4H), 3.5-3.6 (m, 15H), 4.3 (m, 1H), 4.5 (m, 1H), 6.8 (s, 2H); R
- 20 (KBr, cm⁻¹): 3280, 2910, 2850, 1760, 1690, 1640, 1110, 940; HRMS: calculated for C₂₄H₃₈N₄O₇S (M+H) is 527.2539, found 527.2526.

EXAMPLE 10**Method for Preparing a Water Soluble Biotinylation Reagent that is****25 Reactive with Aldehydes, Ketones, and Oxidized Carbohydrates**

- A solution of 0.5 g of biotin-trioxamido-glycolate TFP ester in 50 mL anhydrous THF is added, dropwise, to a solution containing 5 mL anhydrous hydrazine in THF at 0°C. After the addition is complete, the reaction solution is
- 30 allowed to come to room temperature over a 1 hour period. The THF and excess hydrazine are removed under vacuum. The residue is triturated with 100 mL ether and the product (compound 20) is collected by filtration.

EXAMPLE 11**35 Biotinidase Resistant Biotinylation Reagent**

This experiment was conducted to examine the stability of water solubilized, radioiodinated biotin derivatives toward biotinidase degradation in mouse and human serum. Control and Experimental derivatives were synthesized to conduct the study. The biotin derivatives synthesized contained: 1) the biotin moiety; 2) a water solubilizing linker moiety; 3) *p*-iodobenzoate or *p*-tri-*n*-butylstannylbenzoate moieties; and 4) the experimental compounds contained N-methyl or α -methyl moieties to block biotinidase activity. The water soluble linker moiety 4,7,10-trioxa-1,13-tridecanediamine was included in the biotin derivatives to improve their water solubility and it also functioned as a 17Å spacer between the biotin and the benzoyl moieties. Thus, the compounds that were designed to be stable to biotinidase incorporated N-methyl and α -methyl moieties adjacent to the biotin carboxylate group. To measure the biotinidase resistance of the radioiodinated experimental compounds, each was incubated in mouse or human serum at 37°C for 2 hours. Excess streptavidin was then added to bind with the biotin. The solutions were then filtered through a size exclusion centrifugation membrane. Radioactivity that passed through the membrane represented that biotinidase had cleaved the molecule. The results indicated that the biotin derivatives that had no functional group for blocking the biotinidase were rapidly degraded by the enzyme. The biotin derivatives containing the N-methyl-biotinamide functionalities were found to be slightly more stable to biotinidase degradation than the α -methyl biotinamide functionality and significantly more stable than the unprotected control biotin compound.

Other biotinidase blocking groups, such as α -amino acids, can be used in the present invention. Further, the biotin amide can be reduced to an amine. In addition, the pentanoic acid side chain in biotin can be shortened or lengthened. Thus, compounds such as norbiotinamine, homobiotinamine and their isothiocyano derivatives can be used to prepare biotin-containing compounds that are resistant to biotinidase activity.

30 **EXAMPLE 12**

Biotinylated Starburst Dendrimers

In this experiment, a series of biotinylated starburst dendrimers (BSBDs) were prepared and evaluated *in vitro* and *in vivo*. Starburst dendrimers (SBD) of increasing sizes are given the terminology of "generations" as they are built up from "shells". The generations (abbreviated G) used were 0 (G=0; 4 terminal amines); 1 (G=1; 8 terminal amines); 2 (G=2; 16 terminal amines); 3 (G=3; 32

terminal amines); and 4 (G=4; 64 terminal amines). SBD nomenclature and chemistries are provided in a publication by Tomalia and Durst "Genealogically Directed Synthesis: Starburst/Cascade Dendrimers and Hyperbranched Structures", *Topics in Current Chem.*, 165, 193-313 (1993).

5 Five (5) SBDs were biotinylated with 12-N-Biotinyl-(N-methyl) amino-4,7,10-trioxado-decanoic acid tetrafluorophenyl ester 27. (Prepared in Example 4) (BTDT). BTDT is a useful biotinylation reagent as it has a low molecular weight (e.g., 445 g/mol for the conjugated moiety), contains water solubilizing ether functionalities and a N-methyl functionality for stabilization against biotinidase
10 cleavage. The SBDs (G=0,1,2,3 and 4) were obtained as 10-20% by weight solutions in methanol. After removing the methanol under vacuum, the SBDs were reacted with an excess of BTDT 14 in dimethylformamide at room temperature. The crude products were purified by silica gel column chromatography. Mass spectra data confirmed the identify of the compounds prepared. All the SBDs
15 were full perbiotinylated except G=4 (64 terminal amines). From the mass spectra, it appeared that G=4 only added 51 biotin moieties and not the predicted 64. It is likely that longer reaction times and/or elevated reaction temperatures could add more biotin moieties to G=4.

 The BSBDs were radioiodinated so their *in vivo* distribution could be
20 evaluated. Radioiodination was accomplished by reacting sub-stoichiometric quantities of the amine reactive p-[¹²⁵I] iodobenzoate NHS ester with the SBD prior to reaction with the BTDT. The low stoichiometric quantity of the NHS ester was used to assure that only one iodobenzoate was incorporated in the radiolabeled polybiotin compounds.

25 Once the BSBDs had been prepared, an assessment of the number of radiolabeled streptavidin molecules ([¹²⁵I]SAv) that bound each of the BSBDs was made. To accomplish this, an assay was used in which unlabeled BSBDs were bound with immobilized streptavidin (SAv) in polystyrene wells (similar to Example 7), then radioiodinated SAv was bound to the exposed biotin moieties. In the
30 studies, the pmol quantity of [¹²⁵I]SAv bound after saturating the biotin binding sites with unlabeled BSBDs was measured. Those values, shown graphically in Figure 2 were obtained directly from the radioactivity measurements and the specific activity of the added [¹²⁵I]SAv. However, to obtain an estimate of the average number [¹²⁵I]SAv molecules bound with each BSBD (after one addition),
35 an assumption had to be made. That assumption was that the pmol quantities required for 1 equivalent of [¹²⁵I]SAv binding was equal to the amount of [¹²⁵I]SAv bound with the biotin tetramer (G=0). The rationale for using this assumption was

that the value obtained (3.6 ± 0.2 pmol) was very close to that previously obtained for biotin trimers (see Example 7) in the same assay system, and it seems most likely that G=0 would bind with 1 molecule of [125 I]SAv after binding with immobilized SAV. If one uses the 3.6 pmol value for each equivalent of [125 I]SAv bound, the number of equivalents of [125 I]SAv bound with the BSBDs can be readily estimated. By definition, BSBD (G=0), which has four (4) biotin moieties, bound with one equivalent of [125 I]SAv and one equivalent of polymer bound SAV. In the BSBD (G=1), the number of biotin moieties is 8. If two of the biotin moieties on BSBD (G=1) are bound to the immobilized SAV, it might be estimated that 3 additional [125 I]SAv molecules could bind (assuming 2 biotin moieties bound each SAV molecule). Instead, a doubling was seen, binding 2 equivalents of [125 I]SAv. Similarly, with the BSBD (G=2), which has 16 total biotin moieties, 4 equivalents of [125 I]SAv bound in addition to binding with SAV on the plate. No additional increase in the number of [125 I]SAv molecules bound were found with BSBD (G=3; 32 biotin moieties) or BSBD (G=4; up to 64 biotin moieties when perbiotinylated).

The smaller number of [125 I]SAv molecules bound to the SAV-coated plates than had been predicted, based on the number of biotin moieties (pairs) available, might be the result of steric hindrance. This same explanation may account for the lack of increased binding observed for G=3 and G=4 over that observed for the smaller BSBDs. However, one cannot rule out the possibility that, as the BSBD molecules get larger, fewer of them bind with the immobilized SAV. Fewer molecules of the larger BSBDs might bind if: 1) more than one immobilized SAV bound with each BSBD molecule and/or 2) if steric hindrance from the bound BSBD molecules precluded binding of more [125 I]SAv molecules. Another consideration is that after reaching a certain size, more than 2 biotin moieties might bind with each SAV molecule. Through computer modeling, the inventors herein have estimated that it would take a linker of >60Å length (biotin carbonyl carbon to second biotin carbonyl carbon) to bind at opposite faces of SAV. The estimated bond distances between any two biotin moieties in the SBDs G=1-4 appear to be adequate for more than 2 biotin moieties to bind with a single SAV molecule. Irrespective of the explanation for the results obtained, there appears to be no advantages for use of BSBDs larger than generation 2. (G=2).

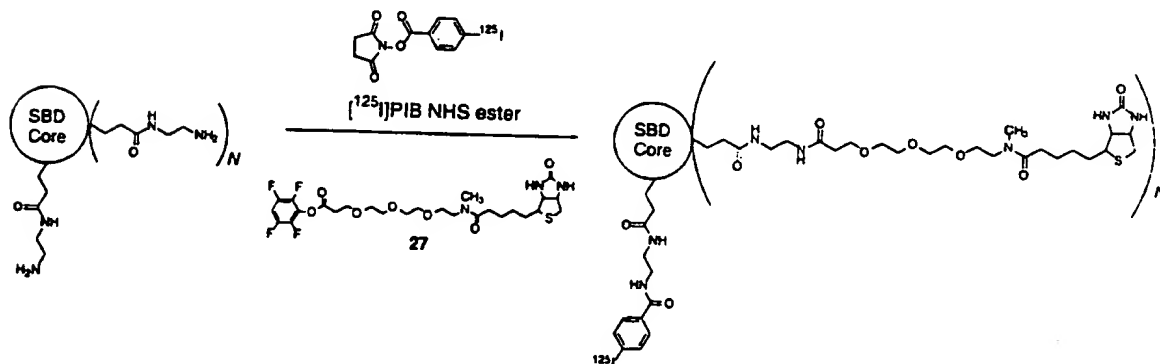
Even though a smaller number of SAV molecules were attached to the immobilized BSBDs than might be predicted, substantial amplification of the amount of radioactivity bound to the SAV coated plates was obtained. From Example 7, it was known that in a single addition, cross-linking with biotin trimers in the same assay system resulted in 4-6 pmol of [125 I]SAv bound per well. Further, it was

demonstrated that a doubling of that quantity (10-12 pmol) of [125 I]SAv was obtained in 4 alternating cycles of reagent additions. However, much larger quantities (16-30 pmol) of [125 I]SAv were bound when biotinylated SBDs (G=2,3, or 4) were used. Indeed, with one addition of the biotin multimers (G=2,3 or 4), there was a 300% increase of [125 I]SAv bound over that of a single addition of the biotin trimers and a 40+% greater quantity bound than obtained previously with four alternating administrations of the biotin trimers. These results support the potential for amplification of tumor targeting of radioactivity using the biotin multimers according to the invention and radiolabeled SAv.

As the ultimate goal is to use the BSBDs for *in vivo* application to tumor pretargeting of radionuclides for cancer therapy, it is important to evaluate the *in vivo* distribution and pharmacokinetics of the BSBDs. Example 7 demonstrates biotin trimers cross-linked SAv in the coated wells, but did not investigate *in vivo* distribution. Therefore, radiolabeled biotin trimers as carriers of radionuclides were included in the below described *in vivo* evaluation. The BSBD, which as 15 biotin moieties (G=2), appeared to be the best candidate for *in vivo* amplification of binding sites, but the intermediate size of BSBD (G=1) made its *in vivo* distribution of interest as well. Although it is envisioned that the perbiotinylated SBDs (G=1 or 2) will not be radiolabeled for *in vivo* use, it was necessary to radiolabel them to assess their biodistribution. It was assumed that these radioiodinated versions of the biotinylated dendrimers would have a distribution similar to the perbiotinylated SBDs.

In this investigation, it was determined that it would be adequate to obtain comparative information about the tissue distribution of the three BSBDs (G=0, 1 and 2) in athymic mice at one time point (e.g., 4 hours post injection).

A generalized reaction of starburst dendrimers (SBD) with [125 I]PIB NHS ester and biotinylation reagent 27 is set forth below:



A				B		
Starting Dendrimer ^a A	MW (amu)	Reaction time (days)	Biotin moieties ^b N	Product B	MW ^c (amu)	Radiochemic al yield (%)
32; SBD, G=0	517	3	3	[¹²⁵ I]35	2077	54
33; SBD, G=1	1430	3	7	[¹²⁵ I]36	4765	28
34; SBD, G=2	3256	3	15	[¹²⁵ I]37	10 140	40

^aThe starburst dendrimer core for each generation (G) is composed of the previous generation, except the terminal primary amines are substituted with two of the groups shown in parentheses in structure A.

^bThis is the maximum number that can be attached under normal conjugation reaction conditions.

^cThe molecular weight has been calculated with the predominant iodine nuclide, iodine-127.

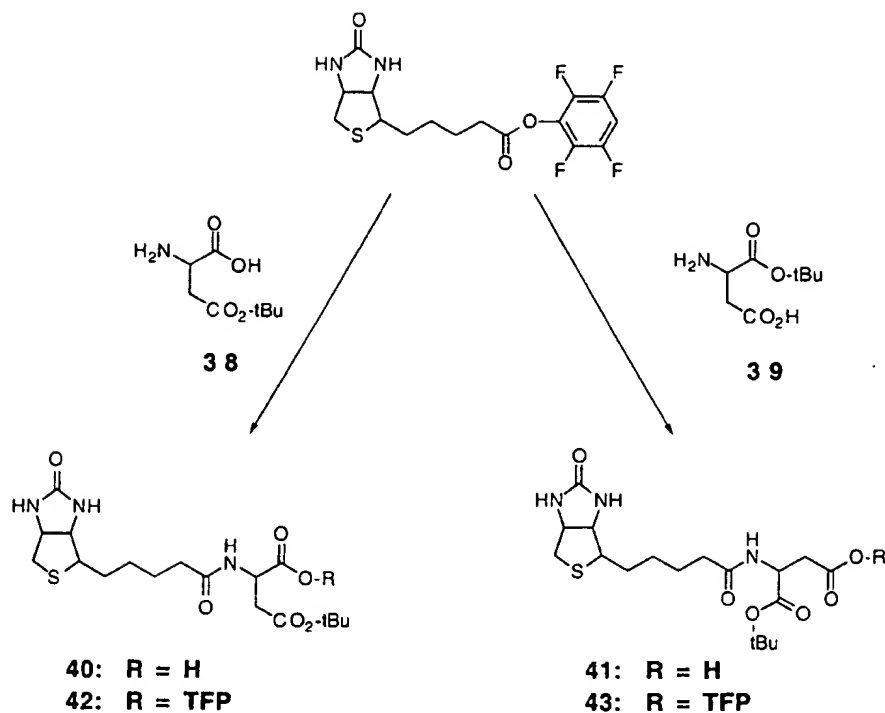
The results of the investigation are shown in Table 1.

Table 1: Distribution of Radioactivity for [¹²⁵I]BSBDs in Athymic Mice^a

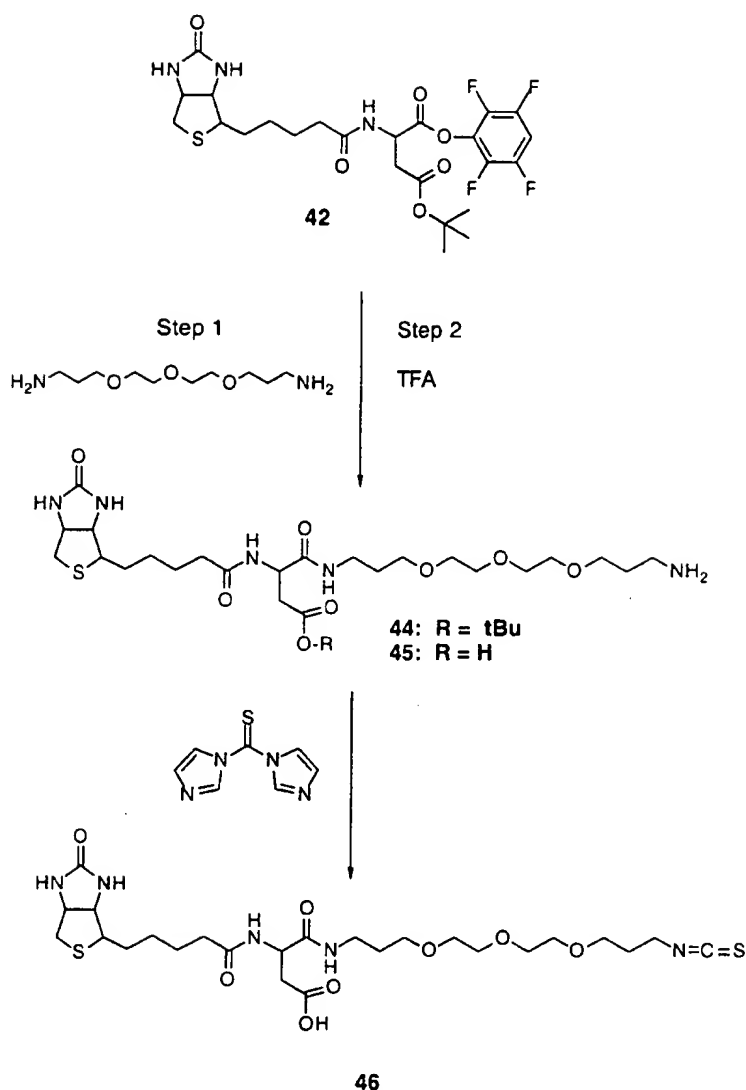
Tissues	[¹²⁵ I]35 ^b	[¹²⁵ I]36 ^c	[¹²⁵ I]37 ^d
blood	0.20 ± 0.04	0.13 ± 0.02	0.20 ± 0.03
muscle	0.04 ± 0.00	0.04 ± 0.00	0.08 ± 0.03
lung	0.26 ± 0.05	0.18 ± 0.01	0.62 ± 0.19
kidney	8.28 ± 2.92	16.95 ± 2.82	47.64 ± 7.08
spleen	0.26 ± 0.11	0.20 ± 0.04	0.70 ± 0.07
liver	1.50 ± 0.10	1.79 ± 0.22	7.06 ± 1.23
intestine	3.77 ± 3.65	0.74 ± 0.66	1.34 ± 1.93
urine ^e	33.39 ± 36.85	9.70 ± 10.25	0.43 ± 0.09
stomach	0.25 ± 0.08	0.14 ± 0.03	0.47 ± 0.11

^aValues shown are average % injected dose / gram ± standard deviation; sacrifice was 4 h post injection; data were obtained for *n* = 5 mice per group; average animal weight, 24.61 ± 2.28 g; all injections were made in

Not furnished at time of publication



The aspartate *tert*-butyl esters **38** and **39** were purchased from a number of commercial sources. Reaction of **38** or **39** with the tetrafluorophenyl (TFP) ester of biotin, provided the adducts **40** and **41**, respectively, in high yields. The TFP esters of **40** and **41** were readily prepared using tetrafluorophenyl trifluoroacetate to give biotin-aspartate conjugates **42** and **43**. Either of these compounds can be reacted with amine (or polyamine) containing compounds (e.g., SBDs) to form multi-biotin-containing compounds. Treatment with trifluoroacetic acid cleaved the *t*-Bu esters to form free carboxylates. While this reaction yields useful biotin-dendrimer compounds, the preferred biotin-dendrimer compounds contain a water solubilizing linker moiety between the core dendrimer and the biotin derivative. Thus, as a preferred example, the synthesis of a biotin-aspartate derivative which also contains a trioxtridecane linker and an isothiocyanate moiety for reaction with amines is depicted below.

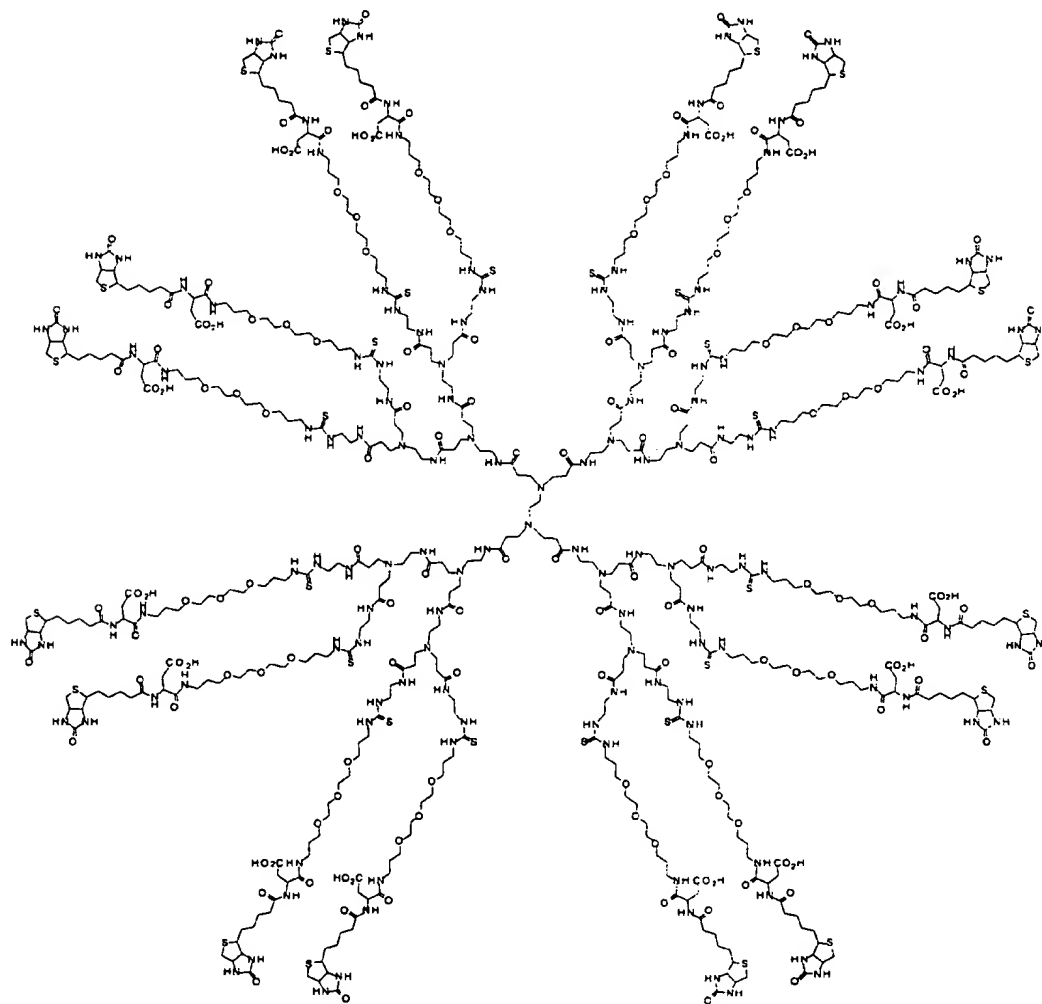


Reaction with biotin-aspartate TFP ester **42** with trioxatridecanediamine yielded the adduct **44**. The *t*-butyl ether was cleaved from **44** with trifluoroacetic acid (TFA) and the amine functionality was converted to an isothiocyanate group with diimidazolethiocarbonyl, to give the desired biotinylation reagent **46**. The same chemistry works with the other biotin-aspartate TFP ester described above.

Under physiological conditions (e.g., pH 7) the aspartate carboxylates deprotonate to yield a negative charged species. These anionic biotin derivatives were then reacted with a starburst dendrimer, generation 2 (SBD, G=2) which has 16 amino groups to react with. After addition of 16 biotin moieties (see Example 12), the net charge on the resulting biotinylated dendrimer was +14. It is believed that this high positive charge causes these compounds to be

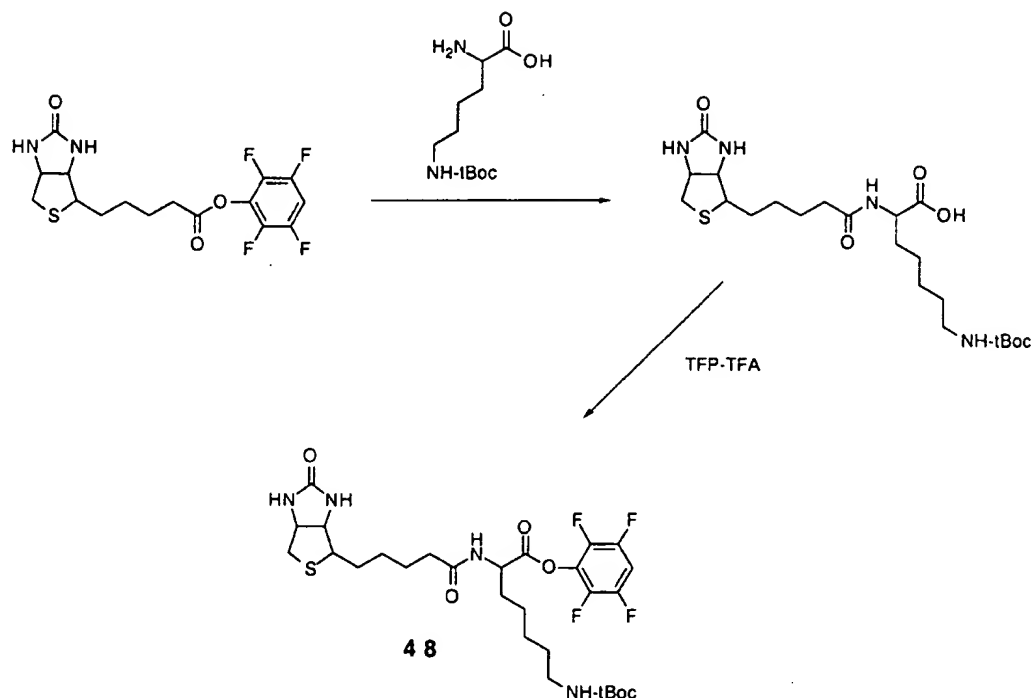
accumulated/retained in the kidney (see Table 1). Reaction of starburst dendrimer, generation 2, with, for example, with biotin aspartate derivative **46** provides the starburst dendrimer **47** that is shown below. Under physiologic conditions, this multibiotin compound has a net charge of -2.

5

**47**

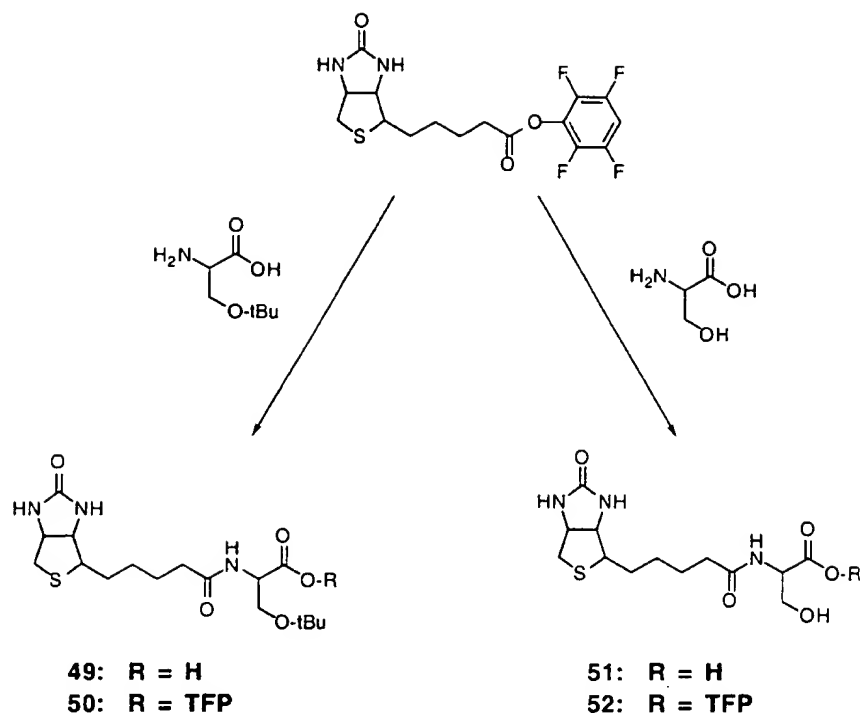
The foregoing depicts biotin derivatives with added negative charges to multi-biotin-containing compounds. Negatively charged cross-linkers (C) (e.g., polycarboxylates) may require the addition of positively charged biotin derivatives to provide more favorable characteristics to the final biotin-containing compound. In such examples, addition of an appropriately protected lysine to biotin to form an

adduct will achieve the desired effect. An example of the synthesis of a biotin-lysine adduct, **48** is provided below.



5

This compound can be conjugated with chemical entities bearing amines or a linker such as the 4,7,10-trioxa-1,13-tridecanediamine, can be added before the reaction with the cross-linker. The t-Boc protecting group can be removed with TFA. The resulting free amine will have a positive charge at physiologic conditions. The net charge of the multibiotin containing compound according to the invention is dependent on the number of biotin-lysine adducts added and in addition to the other charged moieties present. The addition of charged species generally improves the water solubility of multi-biotin-containing compounds. However, alteration of charges on the species may not be desired. Therefore, in certain circumstances, it may be desirable to increase the aqueous solubility of biotin multimers by the addition of another, non-charged species. This can be achieved by preparation of a biotin-serine adduct as shown below.



Two (2) biotin-serine adducts can be prepared, one with a *t*-Bu ether functionality, **49**, and the other with a free hydroxyl moiety, **51**. The tetrafluorophenyl esters, **50** and **52**, can be readily prepared using tetrafluorophenyl trifluoroacetate (TFP-TFA). Choice of these derivatives will be dependent on the specific requirements of subsequent reactions. As in other multibiotin-containing compounds in accordance with the invention, a linker moiety is added prior to the addition to the cross-linker with at least tri-functionality.

An important aspect of the present invention relates to the placement of an α -amino acid between the biotin moiety (B) and the water soluble linker moiety to act as a biotinidase protective group (P). The bulky amino acid groups alpha to the biotinamide bond block the action of the enzyme biotinidase. This is very important for *in vivo* applications of multibiotin-containing compounds in accordance with the invention.

EXAMPLE 14

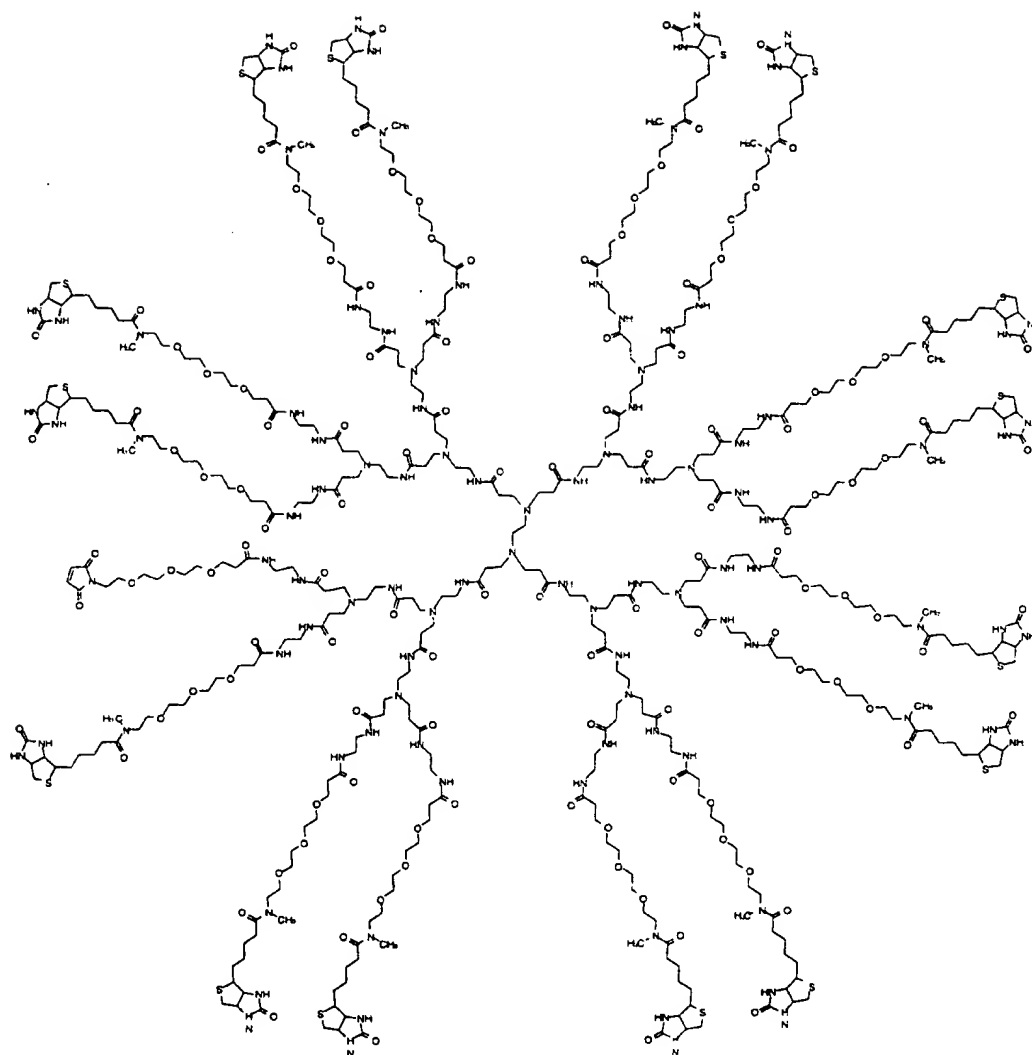
Mono-Substituted Dendrimers as Cross-Linking Agents

The biotin-containing compounds according to the invention which are provided with a targeting or reporter moiety (e.g., chromophore, radionuclide, etc.) should have only one such moiety attached per molecule. This can be

accomplished readily with cascade dendrimers which are built as a branching from one entity (having a protected group for conjugation) but is not readily produced from starburst dendrimers which are symmetrical. However, methods for preparing polybiotin starburst dendrimers can be achieved by limiting the reagents in a manner that only allows one entity to react with the dendrimer.

Reaction of 12-N-phthalimido-4,7,10-trioxadodecanoate TFP ester with amino terminated dendrimers in 0.5-0.75 molar equivalents provided a monosubstituted dendrimer. As an example, the monosubstituted starburst dendrimer, generation 2, (G=2) was monosubstituted with 12-N-phthalimido-4,7,10-trioxadodecanoate TFP ester then perbiotinylated with the biotinylation reagent, 12-N-biotinyl-(N-methyl)amino-4-7-10-trioxadodecanoic acid TFP ester. The N-phthalimido protected, perbiotinylated dendrimer was obtained in good yield. Subsequent cleavage of the N-phthalimido protection group was attained with hydrazine, and the free amine can be converted to any number of conjugation groups (e.g., isothiocyanate, hydroxylamine or maleimide).

A biotinylated starburst dendrimer (G=2) with N-methyl protection and a maleimide conjugation group of the structural formula:



5 This biotinylated starburst dendrimer (G=2) with N-methyl protection and a maleimide functionality is easily conjugated to a monoclonal antibody (targeting protein) using standard disulfide reduction methods (e.g., dithiothreitol or beta-mercaptoethanol) at room temperature and a pH of 6.5. Similar chemistry is used to provide the monosubstituted N-phthalimido protected starburst dendrimer that

10 has multi-biotin-aspartates (15) conjugated. It is important to note that the net charge on this compound is -1 . Minimizing the net charge on perbiotinylated dendrimers conjugated with targeting proteins will minimize the alteration in properties of the targeting protein due to charge. As described previously, the

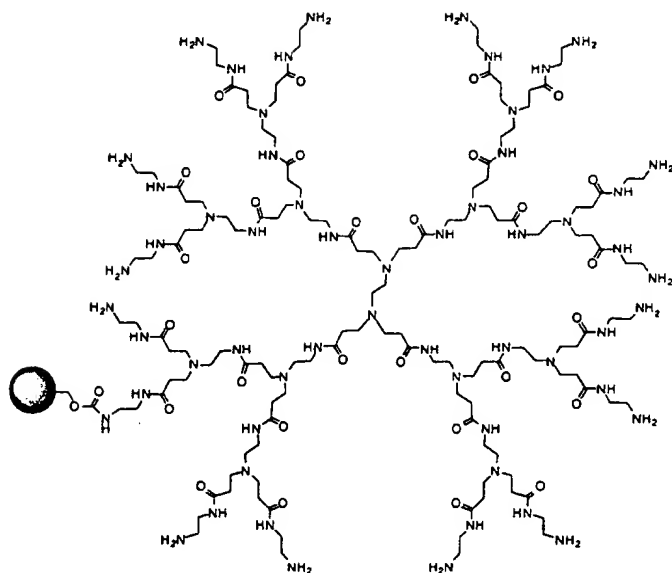
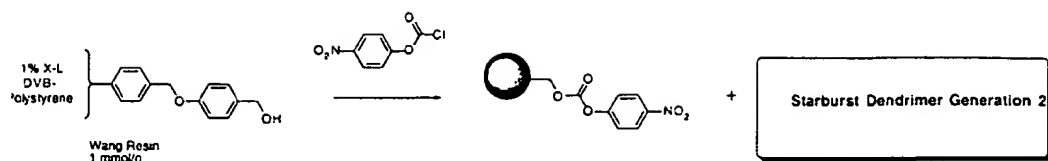
phthalimido protecting group can be removed and the amine can be reacted with another molecule to convert it into a reactive species or it can be directly used to conjugate to another molecule, such as reporter compound (chromophore, radionuclide, etc.).

5

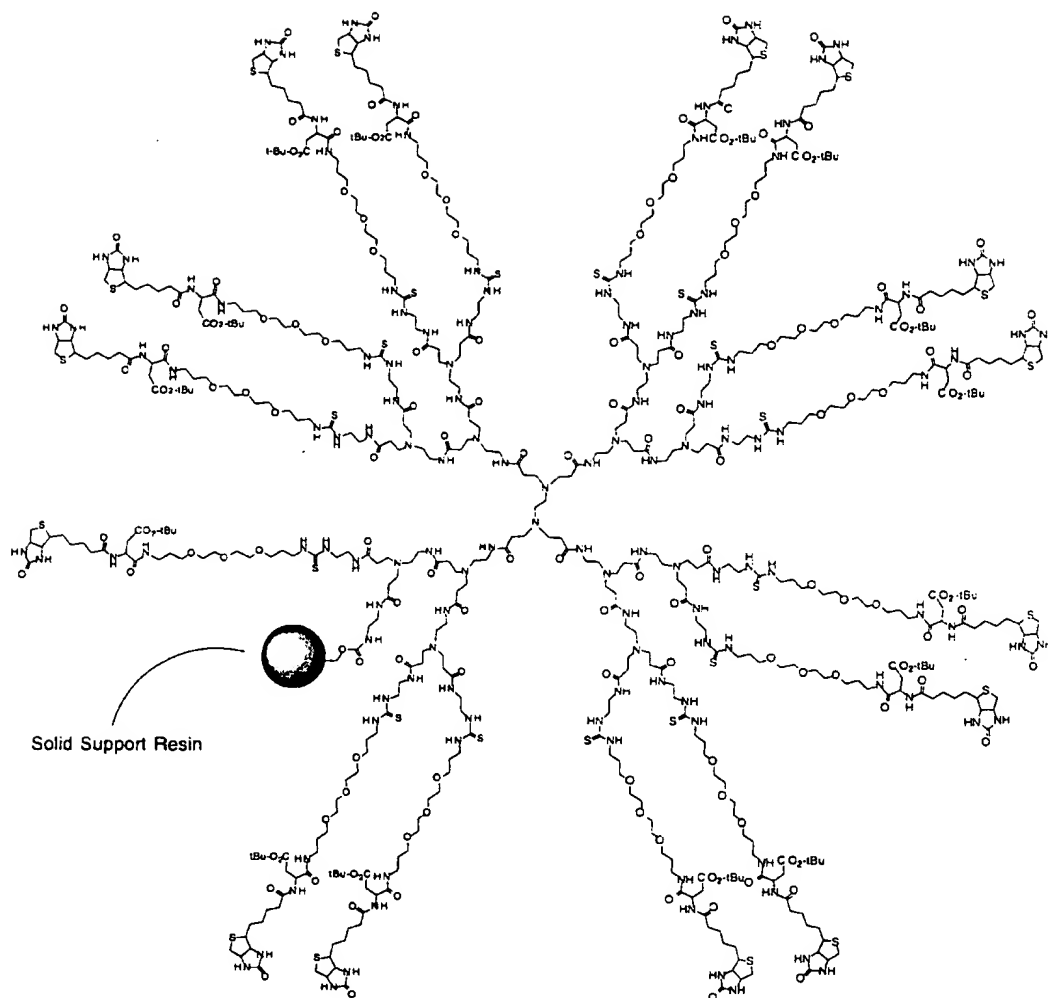
EXAMPLE 15

Solid Support Preparation

An alternative method to prepare the multi-biotin-containing compounds of the invention uses solid support chemistry methodologies. Thus, an amine reactive form of a Wang Resin can be prepared as described by Brady et al. in an article entitled: "Discovery and development of the novel potent orally active thrombin inhibitor N-(9-hydroxy-9-fluorencarboxy)prolyl trans-4-aminocyclohexylmethyl amide (L-372, 460); co-application of structure-based design and rapid multiple analogue synthesis on solid support", *J. Med. Chem.*, 41, 401-406. The amine reactive form of the Wang Resin can be reacted with a large excess of the amine terminated dendrimer to form the adduct as shown below.



An important aspect of this approach is that separation from the non-reacted dendrimer is facile (filtration and washing) and higher ratios of dendrimer to reactive species can be used as the unreacted dendrimer can be recycled. This latter point allows for only obtaining the monosubstituted dendrimer. Perbiotinylation of the polymer bound dendrimer (e.g., SBD, generation 2) with the biotin aspartate isothiocyanate, **46**, is readily accomplished to yield a multi-biotin-containing compound of the structural formula:



Release from the Wang Resin is accomplished with trifluoroacetic acid (TFA).

5

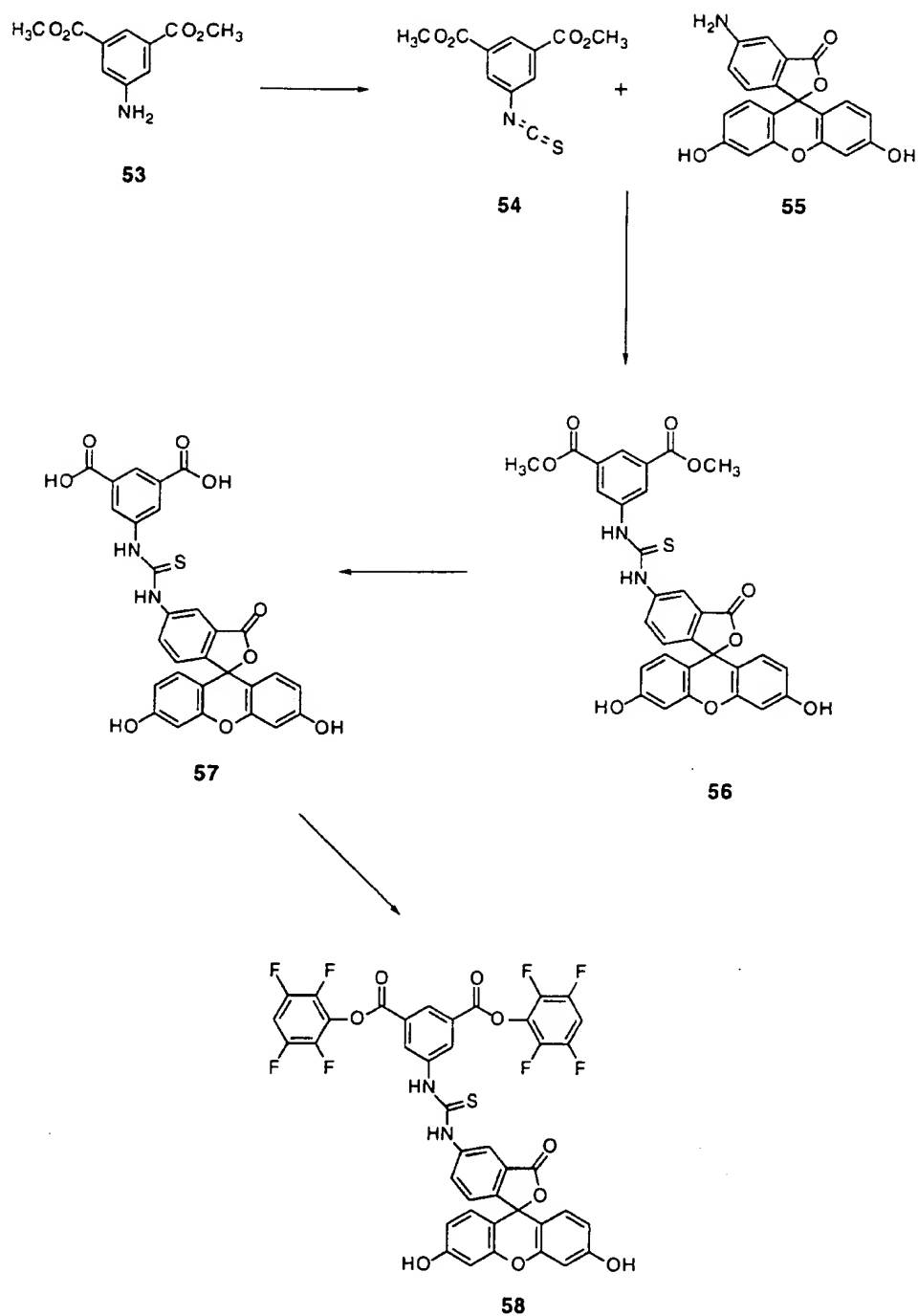
EXAMPLE 16

Detection of Number of Multi-Biotin-Containing Compounds that are Attached to a Targeting Molecule

10 Multi-biotin-containing compounds according to the invention can be conjugated with a variety of carrier or targeting molecules (e.g., antibodies). Based on the structure of the target molecules, reactions will yield a single specific species or a large number of possible reaction products (placed randomly on the molecule). Conjugation with proteins such as monoclonal antibodies is an

example where varying numbers of conjugates can be obtained. Use of the standard HABA method for determining the number of biotin moieties present is not applicable as multi-biotin-containing compounds typically have a large number of biotin moieties on each molecule. Therefore, in some multi-biotin-containing compounds that are conjugated to antibodies and the like, it is desirable to have a reporter group attached to the biotin-containing compound. That reporter group may be a radionuclide carrying moiety (e.g., for I^{125}) or it may be composed of a chromophoric moiety. The latter is preferred in most examples due to the difficulty in storing and working with radioactive materials.

10 Addition of a chromophore to the polybiotinylated molecules can be readily attained with the use of solid support synthesis. A preferred method of introducing the chromophore into the multi-biotin-containing compound is to place it between the multi-biotin moiety (e.g., starburst dendrimer) and the conjugation group (group used to attach the targeting molecule). To accomplish this, the
15 chromophore containing compound should be a homo-or heterobifunctional linking reagent. As an example, the fluorescein chromophore can be used and a homobifunctional linking compound containing that chromophore can be prepared as depicted below.

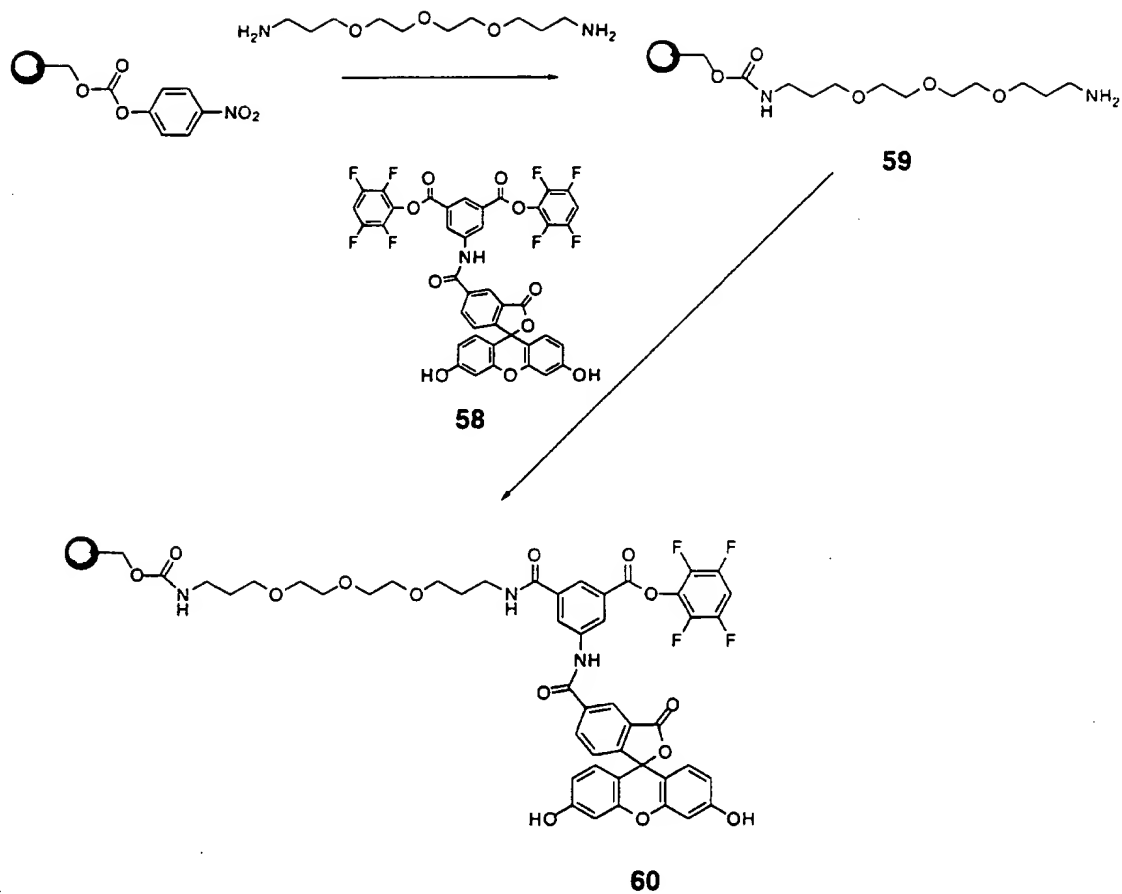


Commercially available aminoisophthalate dimethyl ether **53** is converted to the isothiocyanate **54** and that compound is reacted under basic conditions with
5 aminofluorescein (isomer I) **55** to give the adduct **56**. Following this, the methyl esters are removed and TFP esters are formed to make the desired fluorescein

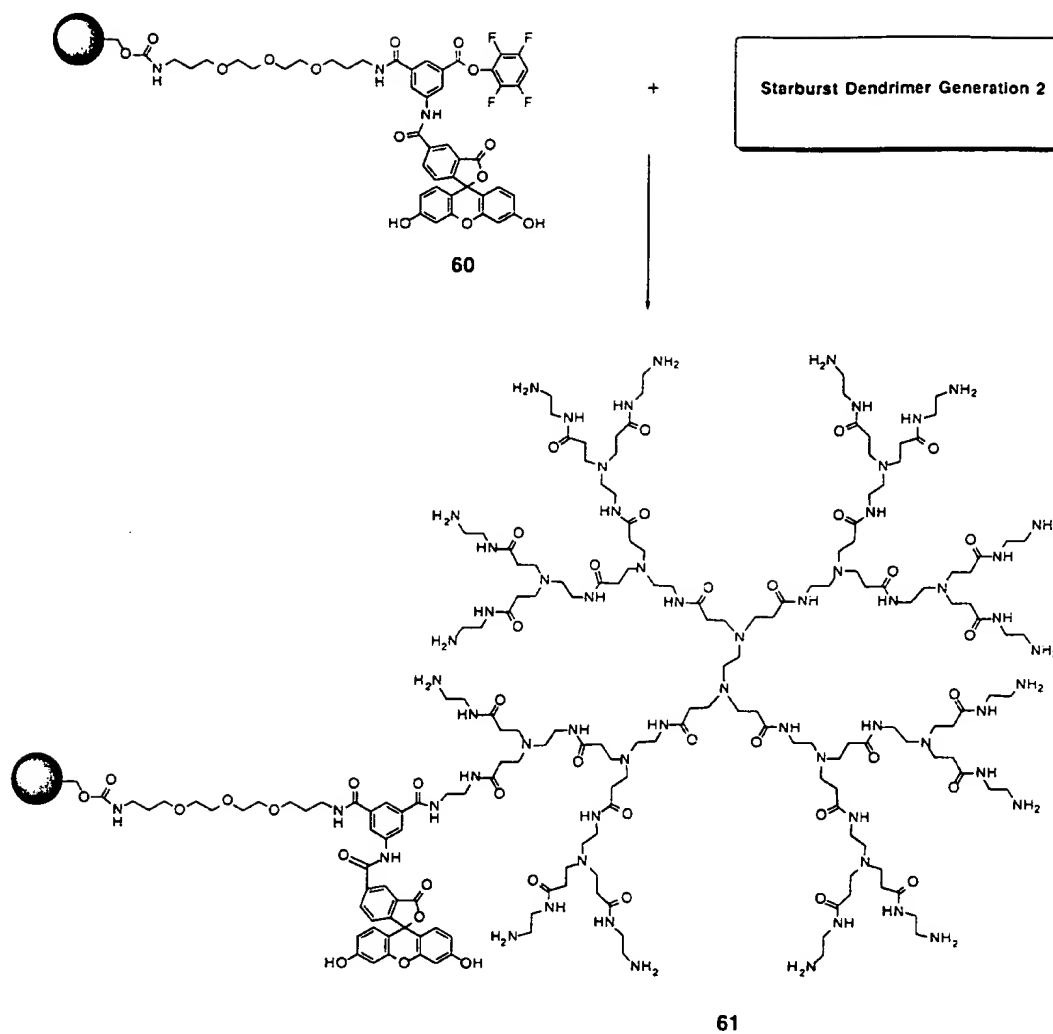
containing homobifunctional linking reagent **58**. Other bifunctional cross-linking reagents containing alternate chromophores can be prepared in a similar manner.

Incorporation of a chromophore (or fluorophore) into the multi-biotin-containing compounds can be accomplished in a step-wise manner using solid support synthesis. The same methods for preparing the multi-biotin dendrimer as previously described in Example 15, can be applied. As an example, the homobifunctional cross-linking reagent **58** which contains fluorescein, can be conjugated to a Wang Resin. After activation, reaction with an excess of 4,7,10-trioxa-1,13-tridecanediamine will provide free amines for conjugation of **58**. To assure that only one amine reacts with **58**, a large excess is used in the conjugation reaction to form polymer supported compound **60**. Importantly, the excess quantities of the trioxatridecanediamine and **58** are readily recovered to be used in other reactions. This reaction scheme is set forth below:

15

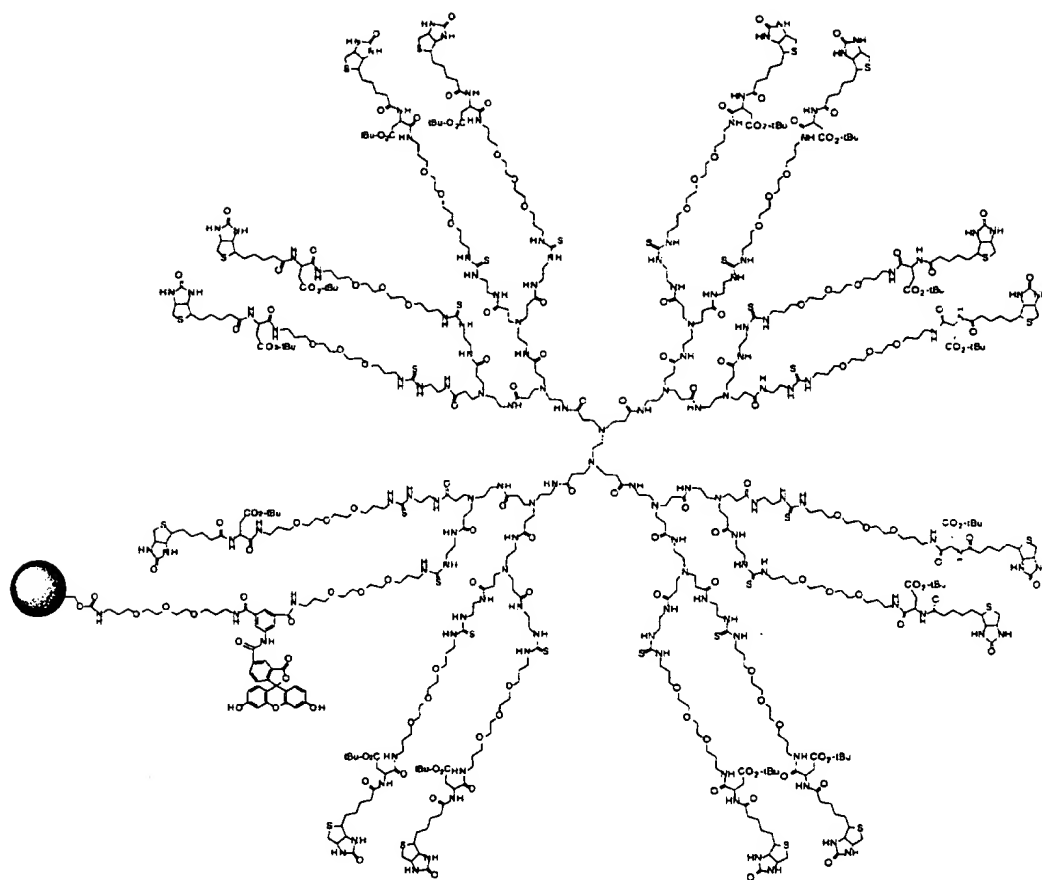


Conjugation of polymer supported **60** with a polyamine (e.g., starburst
5 dendrimer, generation 2) to give the adduct **61** can be achieved as depicted
below.



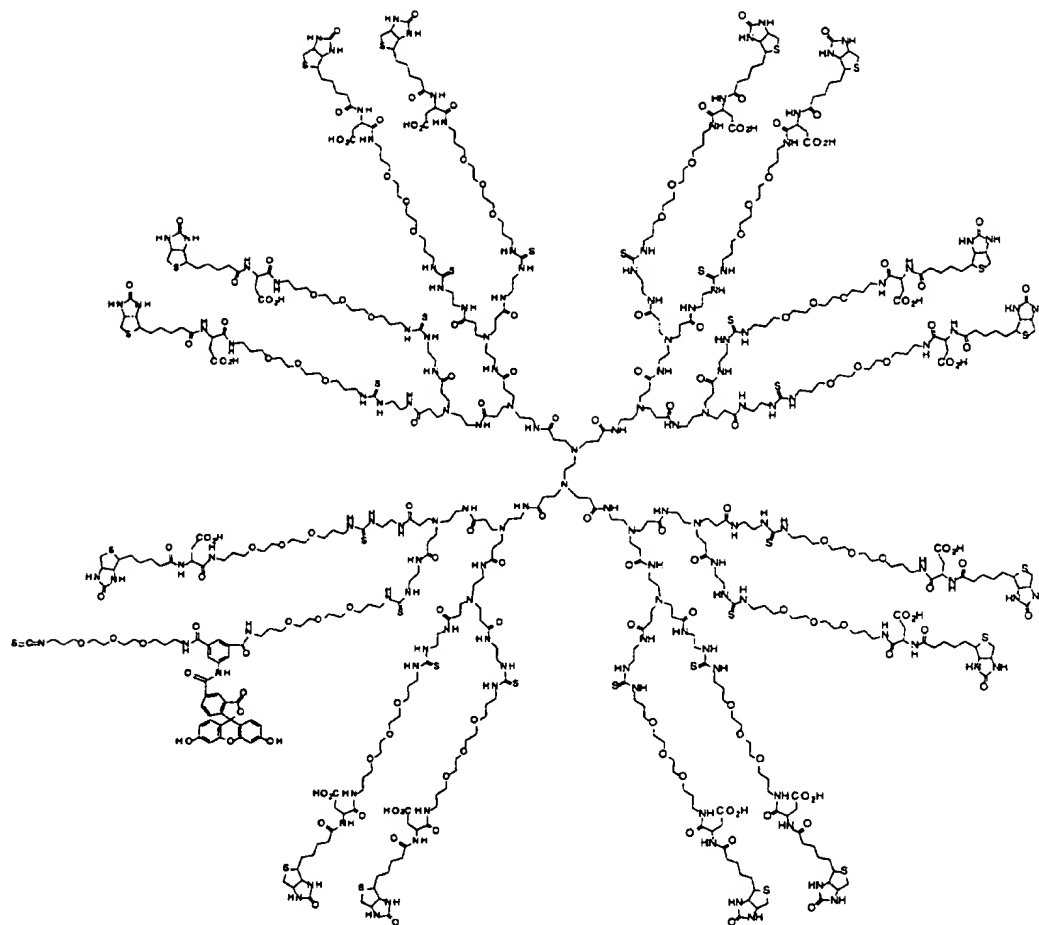
Perbiotinylation with the biotin-aspartate isothiocyanate derivative 46, can be readily accomplished to yield the *t*-Bu ester protected polymer supported compound 62, shown below. Cleavage of the perbiotinylated compound and

5 cleavage of the *t*-Bu esters is accomplished simultaneously using TFA.



62

Once obtained free of resin, the resulting amine can be converted to a conjugation moiety, such as the isothiocyanate, 63, as shown below, or it may be conjugated directly to another molecule.



63

Industrial Applicability

The multi-biotin-containing compounds according to the invention can be produced as discrete compounds that have many applications. Specific embodiments of the invention comprise multi-biotin-containing compounds attached to a carrier molecule or a targeting (receptor binding) molecule. For example, a biotinylated starburst dendrimer can be attached to a cancer cell targeting protein such as a monoclonal antibody for either *in vitro* assays or for *in vivo* diagnostic or therapeutic applications. Attachment of a multi-biotin moiety to a receptor can, in effect, amplify the number of receptors as multiple streptavidin or avidin moieties can be attached to the multi-biotin-containing compound. The concept of amplification of binding sites is one aspect of the present invention and is, in part, the advancement in the state of the art, that the inventors have made.

The medical and scientific communities are constantly searching for improved diagnostic and therapeutic reagents. The present invention provides an advancement in the chemistry of biotin/avidin technology. For example, if a multi-biotin-containing compound according to the invention is used in cancer cell
5 detection through the use of an antibody, amplification is simplified as fewer steps are required. The therapeutic or diagnostic moiety (chromophore, radionuclide, enzyme, etc.) can be attached to the streptavidin or avidin, or it may be introduced via the multi-biotin-containing compound.

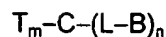
All publications and patents cited hereinabove are incorporated by
10 reference herein, as though fully set forth. Although the present invention has been described with reference to preferred embodiments, workers skilled in the art will recognize that changes may be made in form and detail without departing from the spirit and scope of the invention.

We claim:

1. A biotin-containing compound of the structural formula: $C-(L-P-B)_n$, wherein C is selected from cross-linkers with at least tri-functionality; L is selected from water soluble linker moieties of 6 to 50 atoms in length; P is a biotinidase protective group that blocks biotinidase activity; B is selected from biotin moieties; and n ranges from 3 to 64.
2. The biotin-containing compound according to claim 1 wherein n ranges from 3 to 32.
3. The biotin-containing compound according to claim 1 wherein said cross-linker (C) is selected from at least tri-functional aromatic compounds, starburst dendrimers, cascade dendrimers, polylysine, polyglutamic and polyaspartic acids.
4. The biotin-containing compound according to claim 1 wherein said water soluble linker comprises one or more moieties selected from the group consisting of ethers, hydroxyls, amines, thioethers and thiols.
5. The biotin-containing compound according to claim 1 wherein said biotinidase protective group comprises one or more moieties selected from the group consisting of α -amino acids, N-methyl and α -methyl.
6. The biotin-containing compound according to claim 1 wherein said biotin moiety is selected from the group consisting of biotin, desthiobiotin, biotin sulfone, iminobiotin, alkylated biotins and acetylated biotins.
7. The biotin-containing compound according to claim 1 wherein C is selected from the group consisting of at least tri-functional aromatic compounds, starburst dendrimers cascade dendrimers, polylysine, polyglutamic and polyaspartic acids; L is a water soluble moiety that comprises one or more moieties selected from the group consisting of ethers, hydroxyls, amines, thioethers and thiols; B is selected from biotin, desthiobiotin, biotin sulfone, iminobiotin, alkylated biotins and acetylated biotins.
8. A biotin-containing compound of the structural formula : $C-(L-B)_n$; wherein C is selected from the group consisting of at least tri-functional aromatic compounds, starburst dendrimers, cascade dendrimers, polylysine, polyglutamic and polyaspartic acids; L is a water soluble moiety that comprises one or more moieties selected from the group consisting of ethers, hydroxyls, amines, thioethers and thiols; B is selected from biotin, desthiobiotin, biotin sulfone, iminobiotin, alkylated biotins and acetylated biotins; and n can range from 3 to 64.

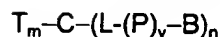
9. The biotin-containing compound according to claim 8 wherein the water soluble linker moiety (L) comprises 4,7,10-trioxa-1,13-tridecanediamine or 12-N-methylamino-4,7,10-trioxadodecanoic acid.
10. The biotin-containing compound according to claim 8 which additionally comprises a moiety adjacent to the biotin carboxylate group which blocks the activity of biotinidase.
11. The biotin-containing compound according to claim 8 which additionally comprises a moiety selected from a diagnostic moiety, a therapeutic moiety and a radionuclide moiety.
12. A non-polymerized biotin-containing compound comprising at least three (3) biotin moieties joined by water soluble linker moieties to a dendrimer, wherein said water soluble linker moieties are 8 to 20 atoms in length.
13. The non-polymerized biotin-containing compound according to claim 12 wherein the water soluble linker moiety is non-ionic and comprises one or more moieties selected from the group consisting of polyether, polyhydroxyl and polyamino compounds.
14. The non-polymerized biotin-containing compound according to claim 12 wherein said biotin-containing compound has a water solubility greater than 0.2 mg/mL at neutral pH and ambient temperatures.
15. The biotin-containing compound according to claim 12 wherein said biotin-containing compound has a water solubility greater than 5 mg/mL at neutral pH and ambient temperatures.

16. A multi-biotin-containing compound of the structural formula:



wherein T is selected from the group consisting of therapeutic, diagnostic and targeting moieties; C is selected from the group consisting of at least tri-functional aromatic compounds, starburst dendrimers, cascade dendrimers, polylysine, polyglutamic and polyaspartic acids; L is a water soluble moiety that comprises one or more moieties selected from the group consisting of ethers, hydroxyls, amines, thioethers and thiols; B is selected from biotin, desthiobiotin, biotin sulfone, iminobiotin, alkylated biotins and acetylated biotins; and n can range from 3 to 64.

17. A multi-biotin-containing compound of the structural formula:

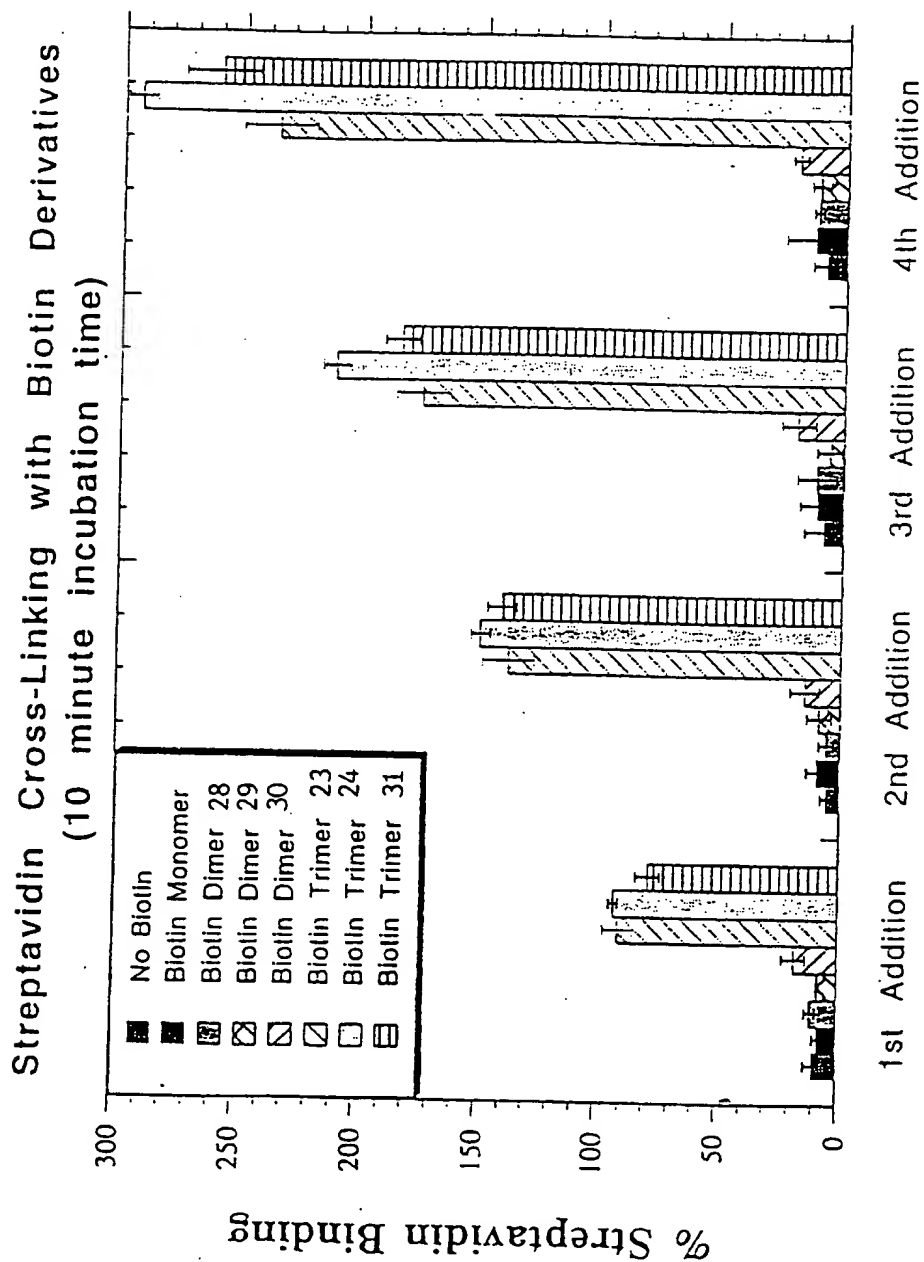


wherein C is a cross-linker of at least tri-functionality selected from the group consisting of tri-functional aromatic compounds, starburst dendrimers, cascade dendrimers, polylysine, polyglutamic and polyaspartic

acids; L is a water soluble moiety of 6 to 50 atoms in length that comprises one or more moieties selected from the group consisting of ethers, hydroxyls, amines, thioethers and thiols; P is a biotinidase protective group selected from α -amino acids, N-methyl and α -methyl moieties; y is 0 or 1; B is selected from biotin moieties; n can range from 3 to 64; T is selected from the group consisting of therapeutic, diagnostic, targeting and active moieties; and m can range from 1 to 5.

18. The multi-biotin-containing compound according to claim 17 wherein m is 1; y is 1; and n can range from 3 to 32.
19. The multi-biotin-containing compound according to claim 17 wherein n ranges from 3 to 32.

The multi-biotin-containing compound according to claim 17 wherein the water soluble linker comprises tri-functional aromatic compounds, starburst dendrimers, cascade dendrimers, polylysine, polyglutamic and polyaspartic acids; L is a water soluble moiety that comprises one or more moieties selected from the group consisting of ethers, hydroxyls, amines, thioethers and thiols; B is selected from biotin, desthiobiotin, biotin sulfone, iminobiotin, alkylated biotins and acetylated biotins; and n can range from 3 to 64.



Biotin Compound Additions

Figure 1

Figure 2

